ISOLATION OF ESTROGEN REGULATED GENES FROM MCF-7 HUMAN MAMMARY CANCER CELLS

1990

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APPROVAL SHEET

Title of Thesis: **"Isolation of Estrogen Regulated Genes from MCF-7 Human Mammary Cancer Cells"**

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Doctor of Philosophy Degree
July 13, 1990

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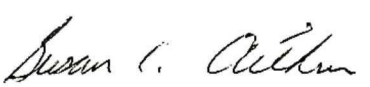

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ABSTRACT

Title of Dissertation: Isolation of Estrogen-Regulated Genes from MCF-7 Human Mammary Cancer Cells

Susan C. Aitken, Doctor of Philosophy, 1990

Dissertation directed by: Daniel R. Schoenberg, Associate Professor, Department of Pharmacology

The established human mammary epithelial cell line MCF-7 has been extensively employed as an *in vitro* model system for the study of hormonally responsive human breast cancer. These cells possess specific receptors for estradiol (E_2) and physiological concentrations of E_2 are required to support cell growth both *in vitro* and *in vivo*. However, although E_2 alters steady-state levels of specific mRNA transcripts, no cloned estrogen-responsive genes are known to regulate MCF-7 cell growth. This investigation has focused on the isolation of estrogen-regulated cDNA clones from cell cultures under conditions that maximize the stimulatory effect of E_2 on cell growth.

MCF-7 cells were maintained under conditions in which cells were either quiescent (estrogen-deprived, E-), or rapidly dividing (estrogen-treated, E+). After seven days the cell number of the E+ cultures increased 3-4-fold while the number of E- cells remained constant. Additionally pulse-labeled E+ cultures incorporated up to 30-fold more [^{3}H]thymidine than E- cultures. E_2 also altered the mRNA expression of three previously isolated cDNAs: pS2 (induced 30-100-fold), 36B4 (unaffected), and gelsolin (decreased 10-fold). R_0t and C_0t analyses confirmed that an E+ cDNA library later constructed in λgt10 accurately reflected the parent RNA population.

The primary library was then screened using a E+ radiolabeled single-stranded cDNA selected by hybridization against a 15-fold excess of E- poly(A) RNA. Northern blots confirmed that all of the final four distinct selected clones (pMT1, pMT2, pMT3, pMT4) were induced by estrogen treatment. pMT1 was subsequently identified as progesterone receptor, a known estrogen-regulated gene.

pMT2 was subcloned into pGEM3Z as two separate EcoRI fragments of 2.2 and 4.5 kb. The cDNA hybridized to a single mRNA species of approximately 11 kb and the sequence of this clone did not correspond to any gene registered in GENBANK. Physiologically relevant concentrations of E_2 increased pMT2 cytoplasmic mRNA levels within one hour whereas the anti-estrogen 4-hydroxy-tamoxifen acted as a partial agonist, suggesting that the effects of estrogen on pMT2 steady-state mRNA levels may be mediated by a classic estrogen receptor mechanism. These clones may prove useful tools in further investigating the mechanism of estrogen regulation of cellular growth.

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by

Susan C. Aitken

Dissertation submitted to the Faculty of the Department of Pharmacology, Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 1990

DEDICATION

This body of work is dedicated to the memory of my parents, Harry Smith Aitken and Elizabeth Locke Aitken, who viewed education as a critical tool enabling each of their children to develop to their full individual potential.

ACKNOWLEDGEMENTS

At this time I would like to acknowledge the work of my individual committee members whose constructive criticisms enhanced the quality of this work and caused me to develop both a more critical and focused approach to scientific research. My thanks to Professor Lewis Aronow for his overview of this study. I am indebted to Dr. Robert Silverman both for his even-handed approach to the entire project and for presenting me with a critical negative control in the course of this investigation. My committee chairman, Dr. Jeffrey Harmon, has consistently been both kind and firm in conveying the necessity of rational and succinct thought in any scientific pursuit. Especially I wish to thank my thesis advisor, Dr. Daniel Schoenberg, for allowing me to work in his laboratory. I may be the first of his graduate students but not the last, and I am sure all will leave his tutelage formidably equipped for the challenges of a scientific career. I am deeply and profoundly grateful to Dr. Marc Lippman, who acted as both a supervisor and as a friend in giving me incredible support and understanding during this sojourne in graduate school. Without his committment to this project and his critical but positive reinforcement, this investigation could never have succeeded.

I specifically wish to acknowledge and thank my fellow graduate student John Moskaitis for performing the sequencing reactions in this investigation. I also deeply appreciate the work done by my advisor, Dr. Daniel R. Schoenberg, in the computer analysis of the sequencing data. My thanks are also extended to Mr. Richard Schrader of the NIH DEC10 facility, who assisted me in the use of MLAB.

Finally I would like to turn to a major support system, the network of graduate students and post-doctoral fellows within the University and particularly within the department of Pharmacology. Special thanks are due to Pam, Helen, Shelly, and Marilyn who have successfully departed, and to John and Lisa who will soon be successfully departing. I have enormously enjoyed working with the various members of Dr. Schoenberg's laboratory, Anna Tate, Mary Beth Martin, Larry Smith, Carol Arras, John Moskaitis, Ricardo Pastori, and Susan Bujak. I sincerely hope these friendships will continue beyond graduate school.

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ABBREVIATIONS

E_2 (estradiol); OH-TAM (4-hydroxy-tamoxifen); TAM (tamoxifen); HR (hormone receptor); ER (estrogen receptor); PR (progesterone receptor); GR (glucocorticoid receptor); VDR (Vitamin D receptor); T3R (thyroid hormone receptor); AR (androgen receptor); MR (mineralocorticoid receptor); RAR (retinoic acid receptor); CRBP (cellular retinol binding protein); MMTV (Mouse Mammary Tumor Virus); HRE (hormone response element); ERE (estrogen response element); PRE (progesterone response element); GRE (glucocorticoid response element); CRE (cAMP response element); SRE (serum response element); RBP (retinol binding protein); LTR (long terminal repeat); HMG proteins (high mobility group proteins); PDGF (platelet-derived growth factor); EGF (epithelial growth factor); NGF (nerve growth factor); TPA (12-*O*-tetradecanoylphorbol 13-acetate); FGF (fibroblast growth factor); TGF (transforming growth factor); IGF (insulin-like growth factor); EGFR (epithelial growth factor receptor); GAP (GTPase-activating protein); PLC (phospholipase C); PKC (protein kinase C); PLA (phospholipase A); IP (inositol phosphate); PI (phosphatidyl-inositol); PC (phosphatidyl-choline); DAG (diacylglycerol); MPF (maturation promoting factor or M phase promoting factor).

INTRODUCTION

I. Historical Background

A number of major scientific contributions preceded the development of the current model of estrogen action. Effects of hormonal milieu on the developmental state of many organs had been noted since the 19th century. Indeed a specific bioassay was developed (i.e. increase in uterine wet weight and vaginal cornification) for a compound secreted in quantity from the ovarian follicle (Astwood, 1938; Kahnt and Doisy, 1928). The subsequent chemical isolation of estradiol established this compound as the chemical mediator of a specific biological response (MacCorquodale, 1936).

By 1940 it was known that estrogens exerted trophic effects in reproductive organs of the rat. Histological studies clearly revealed squamous hyperplasia (cornification) of the vagina (Allen and Doisy, 1924); hypertrophy and hyperplasia of uterus (McLeod and Reynolds, 1938); and hypertrophy and hyperplasia of ductal epithelium of the mammary gland (Lyons *et al.*, 1958). Biochemical changes in uterine wet weight, protein content, and nucleic acid content were documented in the next decade (reviewed by Katzenellenbogen *et al.*, 1979). Enzymologists then began investigations into intermediary metabolism, operating under the hypothesis that steroid hormones modulated specific enzyme activities. For example, increases in the activities of glutamic aspartic transaminase and glutamic dehydrogenase are seen in the rat mammary gland at the time of parturition (Greenbaum and Greenwood, 1954). Some later studies by Villee and co-workers indeed suggested that estradiol acted as a co-enzyme for a specific placental transhydrogenase (Villee, 1961). Theoretically estradiol would alter the intracellular NAD:NADH ratio, thus increasing energy available for anabolic processes in placental tissue. The postulate that specific enzymes constituted the cellular 'receptors' for steroid hormones was widely entertained during this period. However the emergence of the central dogma of molecular biology altered the general direction of these biochemical investigations. In 1957, Mueller proposed that estrogens could increase the rate of many enzyme reactions both by activating preformed enzyme molecules, and by increasing the rate of *de novo* synthesis of enzyme molecules (Mueller, 1957; Mueller *et al.*, 1958).

The proof that hormones modulate levels of particular proteins through *de novo* synthesis has been contingent on technical advances and evolving information in molecular biology. For example, the biochemical basis of any such changes in protein levels remained obscure until it was technically shown that discrete classes of RNA [r (ribosomal), s (soluble, m (messenger)] could be detected on sucrose density gradients (Meselson *et al.*, 1957). The m-class was believed to represent an extremely diverse and unstable population encoding the vast majority of different cellular peptide products (Brenner *et al.*, 1961; Gros *et al.*, 1961). Shortly thereafter, several independent investigators demonstrated that administration of estradiol stimulates mRNA synthesis (Gorski and Nicolette, 1963); Ui and Mueller, 1961; Hamilton, 1964). Further, increases in mRNA synthesis temporally precede increases in net protein and DNA synthesis (Hamilton *et al.*, 1965). Subsequently, the concept of gene activation and repression was formally introduced as a working model for investigations into steroid hormone action (Jacob and Monod, 1961). The insect hormone ecdysterone was shown to modulate the expression of specific genes at the DNA level (chromosome puffs), and to induce the production of a specific gene product (DOPA decarboxylase) through an RNA intermediate (Karlson and Sekeris, 1966).

At approximately the same time, several investigators were independently attempting to characterize the intracellular mediators of such estrogen effects. First, the synthesis of radiolabeled estradiol led to the observation that this material accumulates differentially in known biological target tissues (Glascock and Hoekstra, 1959; Jensen and Jacobson, 1962). Secondly, physical techniques (density gradient analyses) were used to identify a specific intracellular estrogen-binding protein (Jensen *et al.*, 1967; Toft and Gorski, 1966). The presence or absence of this molecule predicted both the biological response to estradiol, and the cellular accumulation of estradiol (Jensen and DeSombre, 1972)

The concept that such receptors act as repressors or activators of gene transcription has since become the chief operative tenet of steroid endocrinologists. Evidence that estrogens modulate the expression of specific gene products, and that ER mediates this effect, has accumulated in a number of systems (reviewed by Strobl and Thompson, 1985). However estradiol has frequently been perceived as both a trophic and developmental hormone. The rat uterus and mammary gland have been chiefly used to examine the effect of estradiol on cell growth. Conversely the chick oviduct and the liver of various egg laying species have been most useful for studies of so-called differentiation functions. To date most studies of gene expression have focused on regulation of high abundance, highly induced gene products (vitellogenin, ovalbumin, etc.) in liver and oviduct. Considerations of abundance and basal levels of expression of hormone-induced products have greatly limited similar mechanistic studies on the estrogenic regulation of growth.

II. Mechanism of Steroid Hormone Action

Current theory concerning the mechanism of control of gene expression invokes change in the synthesis of specific proteins due to an alteration in the steady state concentrations of their cognate mRNAs. In many cases these responses are mediated through a class of intracellular proteins which bind to DNA and alter the transcripion of specific genes by RNA polymerase II.

Three general motifs are now believed to function in confering sequence specificity to the interaction of such proteins with DNA (Ptashne, 1988). The first is a helix-loop-helix (HLH) configuration usually associated with prokaryotic regulators of gene expression, but also identified in eukaryotes (Murre *et al.*, 1989a; Murre *et al.*, 1989b; Schleif, 1988). The general structure is believed to be an amphipathic α-helix associated with DNA binding and flanked by acidic residues involved in protein-protein interactions. Recently the "leucine zipper" hypothesis formulated by McKnight and colleagues has suggested a second chemical basis for the interaction of transcriptional regulatory proteins with DNA (Landschulz *et al.*, 1988). This model specifically predicts the method of homo- and hetero-dimerization of a class of DNA binding proteins represented by Myc, Jun and Fos oncogene products, as well as the transcription factors GCN4 and C/EBP. The third motif, the zinc finger first identified in TFIIIA (Miller *et al.*, 1985; Evans, 1988), has since been observed in a large number of eukaryotic gene regulatory proteins, including the superfamily encompassing steroid and thyroid homone receptors (Evans and Hollenberg, 1988).

The genes for five major classes of steroid receptors, GR (Hollenberg, 1985), ER (Walter *et al.*, 1985), PR (Jeltsch *et al.*, 1986; Coneeley *et al.*, 1986), AR (Chang *et al.*, 1988; Lubahn *et al.*, 1988), and MR (Arriza *et al.*, 1987) are cloned. Related receptors for thyroid hormone [T3R (Weinberger *et al.*, 1986; Sap *et al.*, 1986)], retinoic acid [RAR

(Giguere *et al.*, 1987; Petkovich *et al.*, 1987)], retinol [cellular retinol binding protein or CRBP (Demmer *et al.*, 1987)], and vitamin D3 [VDR, (McDonnell *et al.*, 1987)] are also identified. These genes, which share significant homologies, constitute a superfamily of regulatory proteins, possibly originating from a primordial receptor gene (Green and Chambon, 1986). Closely related genes to the original receptor species have also been reported. For example, Evans and colleagues have isolated two clones designated hERR1 and hERR2 with partial sequence homology to ER (Giguere *et al.*, 1988). hERR1 is expressed as a fairly abundant 2.6 kb mRNA in all rat and human tissues, especially in the nervous system; hERR2 is present at 1-10% of hERR1 levels in a few selected tissues. No class of ligands is yet known to bind to translation products of these two mRNAs.

Cloning of receptor genes has made it possible to reconstitute both *in vivo* and *in vitro* systems to examine both the physico-chemical properties of the molecule which define its function, and the transcriptional and post-translational (i.e. transformation) control of receptor activity. Structural comparisons, in conjunction with functional assays, define specific modular protein domains responsible for DNA binding, hormone binding, and trans-activation of gene expression (Godowski *et al*, 1987; Hollenberg *et al.*, 1987). The precise mechanism by which steroid hormones alter levels of mRNA is not established, although specific receptor binding sites on DNA are at least partially characterized (Anderson, 1985). Further, a functional link between *in vitro* binding of hormone receptor complex to specific DNA sequences and *in vivo* changes in gene transcription is documented in the case of glucocorticoid regulation of MMTV (Pfahl *et al.*, 1983) and tryptophan oxygenase (Danesch *et al.*, 1987).

The molecular basis for selective transcriptional activation by steroid hormones clearly involves interaction of HR complex with specific nucleotide sequences termed HREs. A consensus sequence for ER, **AGGTCANNNTGACCT**, has been derived from gene transfer experiments and receptor-DNA binding studies. The palindromic 13 bp

ERE sequence behaves as an enhancer, operating on a cis-linked promoter at a distance and independent of orientation. The ERE confers estrogen responsiveness to a heterologous promoter and purified ER binds specifically to ERE-containing DNA restriction fragments (Klein-Hitpass *et al.*, 1986). Nuclear extracts of estrogen-treated *Xenopus laevis* liver support the estrogen-dependent induction of transcription of an ERE-vit-CAT construct *in vitro* as measured by primer extension, and the dose requirement for estradiol to produce active extracts are consistent with ER mediating this response (Cortesy *et al.*, 1988). Methylation of corresponding guanine residues on both strands of the ERE prevents binding of hER·estradiol complexes and hER·4-hydroxy-tamoxifen complexes, possibly suggesting a direct interaction of ER with specific nucleotide residues within the ERE (Kumar and Chambon, 1988; Klein-Hitpass *et al.*, 1989).

Glucocorticoid (Tsai *et al.*, 1988), progesterone (Thevany *et al.*, 1987; Guiochon-Mantel *et al.*, 1989), and estrogen (Kumar and Chambon, 1988; Klein-Hitpass *et al.*, 1989) receptors appear to bind their respective hormone responsive elements (HREs) as dimers. In the case of estradiol, hormone is important in inducing the formation of receptor dimers in solution and apparently prior to DNA binding. Constitutive binding of both ER and GR to DNA is observed when the receptor hormone binding domain is deleted; however estradiol is required for binding of wild type ER to the ERE, and that binding is associated with dimerization. The hormone-binding domain also increases the stability of the hER-ERE complex.

In vivo, estradiol binds to ER and increases binding affinity for non-specific double-stranded DNA 7-fold while elevating affinity for the specific double-stranded DNA ERE 17-fold (Lanigan and Notides, 1989). Transitory strand separation and supercoiling are thought to ensue in the region of the ERE. ER then transposes preferentially to the coding strand with a 60-fold increase in affinity, stabilizing the single-stranded configuration. It has also been suggested that steroid hormone receptors recognize specific

DNA sequences by a scanning mechanism (Schauer *et al.*, 1989). Approximately 85% of receptor molecules bind to purified DNA *in vitro* whether or not hormone is present; however, binding of hormone to either GR or PR increases both the on rate (2-5-fold) and the off rate (10-20-fold) of receptor binding to non-specific DNA. Receptor complexed to anti-hormone binds with kinetics intermediate between that of hormone-free or hormone-bound receptor (no effect on k_a but a 5-fold increase in k_d). An increase in these kinetic constants hypothetically could allow more rapid recognition of sequence specific DNA.

The ERE's for different genes do not appear to be structurally or functionally identical. One known ERE in the prolactin gene is localized at -1581 to -1568; a second ERE with only 80% homology to the consensus sequence is detected at -1722 to -1709. Surprisingly the second has a higher affinity for ER (Lanigan and Notides, 1989). Chambon and associates have shown that the ERE of the human pS2 gene is an imperfect palindrome which is less effective than the consensus sequence in conferring estrogen inducibility in a reconstituted system (Berry *et al.*, 1989). The amount of ER present may also limit estrogen responsiveness; the stimulation of pS2 transcription by estradiol in transient assays in HeLa cells is dependent on the efficiency of expression of co-transfected ER (Roberts *et al.*, 1988). Since EREs display such heterogeneity and the level of active ER dimers may be very sensitive to both estradiol and ER concentrations, considerable variation is to be expected in the promoter activity of hormonally sensitive genes.

In the above context, two classes of hormone-regulated genes may exist in *Xenopus* liver (McKearin and Shapiro, 1988). The first class, typified by vitellogenin, requires exogenous hormone and high levels of loaded ER complex to induce and maintain expression. The second class (ER and RBP) show persistent long-term response and have a lower threshold to loaded hormone-receptor complex. The long term induction of retinol-binding protein and ER mRNAs is due to a specific long-term increase in the rate of RBP and ER mRNA transcription believed to be mediated by the classic ER (Barton and

Shapiro, 1988). Schoenberg and colleagues have also suggested (based upon the differential effects of estrogens and anti-estrogens) that different mechanisms may be involved in the estrogenic control of vitellogenin and ER gene transcription (Riegel *et al.*, 1987a). The mechanism of this differential response is unclear; both transcriptional and post-transcriptional mechanisms could be conjectured.

The function of an ERE also appears to be partially dependent on the neighboring enhancer/promoter environment. Indeed multiple hormone receptor binding sites have frequently been observed in steroid-regulated genes. *In vitro* studies confirm functional cooperativity between two binding sites for the GR in the regulatory region of the tyrosine amino transferase and the tryptophan oxygenase genes (Jantzen *et al.*, 1987; Danesch *et al.*, 1987). The transcriptional enhancement of transcription by GR constructs in a cell free system is also dependent on the presence of one or more functional GREs (Freedman *et al.*, 1989).

In many cases different HREs are involved in gene regulation. For example, the lysozyme gene responds to several steroid hormones, including estrogens, progestins and glucocorticoids. Four specific contact points for PR exist in the 5' region (Von der Ahe *et al.*, 1986). Binding of GR in this same general area produces a somewhat different DNase I protection pattern, and three contact points are predicted; one of these is shared with PR. PR and ER appear to function synergistically to activate transcription of the chicken vitellogenin B2 gene (Cato *et al.*, 1988). Similarly, a neighboring strong ERE and weak GRE are separated by two helical turns in the chicken vitellogenin II gene. Both estradiol and glucocorticoids increase transcription, and in concert, elicit a synergistic increase in gene activity. In contrast, if a strong GRE and strong ERE are coupled, an additive effect is observed (Ankenbauer *et al.*, 1988). Experiments utilizing two copies of paired GRE/PRE constructs fused to a TK-Cat reporter indicate that glucocorticoid and progesterone HREs synergistically affect gene transcription (Tsai *et al.*, 1989). Parallel

synergism is seen in both transcriptional activation and DNA binding; the K_a of the second dual steroid response element for GR or PR is greater than that seen with a single dual HRE.

The above data suggest that multiple copies of steroid response elements may increase sensitivity to, or heighten, the response to hormonal treatment. In addition there is abundant evidence that several different types of cis-acting regulatory sites can be found in the vicinity of steroid regulated genes (Schule *et al.*, 1988a; Schule *et al.*, 1988b; Strahle *et al.*, 1988). For example, ovalbumin or conalbumin 5' flanking sequences have been fused to an SV40 T antigen reporter and microinjected into primary liver or oviduct cultured cells (Dierich *et al.*, 1987; Gaub *et al.*, 1987). The organization of the oviduct ovalbumin regulatory region apparently reflects at least three different elements; 1. a steroid independent constitutive element (-56 to +1) which requires a positive cell-specific trans-acting factor; 2. a negative regulatory element specifically repressing the oviduct ovalbumin promoter (-295 to -495); and 3. a steroid regulatory element (-132 to -425).

Recent investigations in both prokaryotic and eukaryotic systems implicate protein-protein interaction between multiple transcriptional regulatory proteins in the final formation of a transcription initiation complex with RNA polymerase II. At least two additional factors, COUP and S300-II, are required for efficient transcription of the chicken ovalbumin gene (Sagami *et al.*, 1986). Whereas S300-II demonstrates little specificity, COUP appears to be an additional member of the estradiol/ thyroid hormone/ vitamin D receptor subfamily (Wang *et al.*, 1989). Estrogen treatment induces the formation of several protein-DNA complexes in the *Xenopus laevis* vitellogenin gene and the chicken vitellogenin B1 gene. Not all of these involve the estrogen receptor or an ERE; the role of the other protein-DNA complexes is unclear (Heggler-Bordier *et al.*, 1987; Cato *et al.*, 1988). Similar modes of action have been proposed for other steroid receptors. PR is thought to facilitate the formation of stable preinitiation complexes (Klein-Hitpass *et al.*,

1990). Further, the positive glucocorticoid induction of transcription of genes linked to the MMTV promotor is believed to be due to the receptor-mediated creation of a transcription factor complex involving new binding of two putative transcription factors (NF1, NF2) at the promoter, rather than activation of a preexisting complex (Cordingley *et al.*, 1987).

Evidence derived from several experimental systems also suggests that DNA binding proteins may directly compete for DNA binding sites. Glucocorticoids are thought to negatively regulate the α subunit of the gonadotropin hormones in the placenta by interfering with the binding of other transcription factors to the cAMP response elements (-146 and -111) in the 5' region of that gene (Akerblom *et al.*, 1988). Full expression of the α subunit requires either induction of the CREs with cyclic AMP, or the presence of a tissue specific enhancer (tse, -224 to -136) coupled to the two CREs, and no effect of glucocorticoids is seen in the absence of full induction. Similarly, the distal enhancer region of the prolactin gene in the pituitary gland regulates response to cAMP, TRH, EGF and estradiol (Day and Maurer, 1989). Both positive and negative estrogen regulatory elements appear to control gene expression and require separate and distinct structural elements of the estrogen receptor (Adler *et al.*, 1988). Further, a general but tissue specific positive transcription factor termed pit-1 binds to DNA sequences which overlap the multiple negative estrogen regulatory elements.

It has become clear that steroid hormone responses at the level of transcription are mediated by either concerted action of repeated nucleotide motifs which constitute hormone response elements (MMTV, Toohey *et al.*, 1986; vitellogenin, Ankenbauer *et al.*, 1988; TAT, Jantzen *et al.*, 1987), and/or cooperation or interference with binding sites for other transcriptional factors in the near vicinity (MMTV, Cordingley *et al.*, 1987; tryptophan oxygenase, Danesch *et al.*, 1987). This type of interaction appears to provide a means for "fine tuning" the regulation of genes known to respond to a number of different positive and negative stimuli.

III. Unresolved Issues

A. Chromatin Organization

Tissue specific gene expression, temporal programming of gene activity, and the memory effect [increased and more immediate hormonal induction of RNA synthesis (i.e. vitellogenin and ovalbumin)] seen after an initial exposure to hormone, are not readily explained by simple models of receptor interaction with DNA (Ryffel *et al.*, 1977). DNA binding proteins may alter transcriptional efficiency by a variety of mechanisms: 1.'tracking'; 2. formation of a DNA loop by bringing two sites together; 3. distal actions through influences on DNA topology (Wang and Giaever, 1988). For example, modification of nucleosomal organization and of DNA conformation is associated with changes in the supercoiling and the writhe, twist and linking numbers of specific DNA regions. Nucleosomal positioning alters the function of cis-acting DNA elements *in vivo* (Simpson, 1990). In yeast, nucleosome loss appears to activate downstream promoters in the PHO gene (Han and Grunstein, 1988). A phased nucleosome array is seen when the gene is repressed, nucleosomes are displaced on gene activation, and the loss of nucleosomes allows constitutive gene expression regardless of the presence or absence of the UAS elements.

A number of studies have been directed toward determining the effect of steroid hormones on the chromatin environment of specific genes *in vivo*. Hormonally-induced alterations are seen in the 3' region of the chicken ovalbumin gene (Bellard *et al.*, 1986); also, changes in chromatin organization of the chicken vitellogenin II gene are associated with primary or secondary estrogen treatment (Burch and Evans, 1986). The MMTV-HRE confers precise nucleosome positioning (phasing) on minichromosomes (Perlman and Wrang, 1988). In the presence of glucocorticoids, a hypersensive site containing the GR

binding site is generated over the MMTV promoter region. Based on the previously documented mapped phasing in the LTR, this site corresponds to the 'inferred' position of a nucleosome (Richard-Foy and Hager, 1987).

It has been suspected that methylation of CpG residues may be involved in the structural ordering of chromatin (Murre *et al.*, 1989; Cedar, 1988); however the mechanism is unclear. Two non-histone proteins (NHP-1 and NHP-2) bind to the ERE of the chicken vitellogenin gene approximately 600 bp upstream of the transcription initiation site (Feavers *et al.*, 1987). Neither protein is tissue or species specific. The presence of NHP-1 enhances the binding of ER to the ERE 8-fold; however, methylation of both strands of the ERE decreases NHP-1 binding by 60%. In contrast, methylation of cytosine residues at the recognition site of the general transcription factor SP1 does not affect either SP1 binding or transcriptional activity (Harrington *et al.*, 1988). Whether methylation is a common mechanism affecting the binding of other proteins, including histones, HMG proteins, matrix proteins, elements of the transcription complex such as DNA polymerase or topisomerase II, and transcriptional regulators in general is unknown. Hormones can alter both the methylation and DNase sensitivity of steroid regulated genes. Estrogen treatment produces a selective, strand specific demethylation of four CpG residues (two within the ERE) about 600 bp upstream of the transcription start site of the chicken vitellogenin II gene (Saluz *et. al.*, 1986). Demethylation correlates with both the appearance of DNAse I hypersensitive sites and the induction of vitellogenin mRNA synthesis. However these sites remain unmethylated even after cessation of gene transcription. Further study of this gene indicates that an additional CpG at position +10 (a tissue-specific regulatory region) is fully methylated in DNA isolated from tissues that do not express the avian vitellogenin gene, but is unmethylated on both strands in the liver of mature hens and estrogen-treated roosters (Saluz *et al.*, 1988). In roosters this CpG site (site B2) becomes demethylated and hypersensitive to DNAse I following estrogen

treatment. Gel shift and *in vitro* DNAse protection assays indicate that a protein present in rooster liver nuclear extracts binds only when that sequence is methylated. Demethylation again coincides with gene expression and site B2 appearance. Such reports suggest that methylation could represent a mechanism for the memory effect; however, neither the maintenance of induced 5' hypersensitive sites nor the methylation of the ERE correlates with the presence or absence of memory in chickens previously treated with estradiol (Burch and Evans, 1986). Although epigenetic phenomena such as methylation may indeed contribute to the specificity of steroid hormone action (reviewed by Anderson, 1985), the mechanism of such action remains unresolved.

B. Modulation of ER Activity

Several steroid hormone receptors appear to be predominantly nuclear proteins (ER, T3R, PR) in the absence of their cognate ligands (King *et al.*, 1984; Welshons *et al.*, 1984, Denis *et al.*, 1988). Regardless of inital localization, all classes of steroid receptors are converted to molecules with increased affinity for nuclei, chromatin, and DNA in a process termed 'activation' or 'transformation' (reviewed by Grody *et al.*, 1982). The presence of hormone is critical in this process. The interaction of the M_r 90,000 heatshock protein (hsp90) cytoplasmic protein with several classes of receptors may affect receptor transformation as well, although the precise functional significance of the hsp90 remains unclear (Riehl *et al.*, 1985; Smith *et al.*, 1990).

Auricchio and co-workers have demonstrated that phosphorylation state appears to be at least one intracellular post-translational mechanism of regulating estrogen receptor activity. In their model, a specific Ca^{++}/calmodulin-dependent cytosolic protein kinase phosphorylates ER exclusively on tyrosine; conversely a nuclear phosphatase dephosphorylates ER complex, hormone is released, and further hormone binding

capability is lost. Phosphorylation is a prerequisite for hormone binding. Estradiol has since been shown to stimulate tyrosine phosphorylation and hormone binding activity of ER in a cell-free system (Auricchio *et al.*, 1987). At present the subject of steroid receptor activation is under active investigation; further progress may be dependent on the development of antibodies directed against specific epitopes on the receptor molecule. The use of cloned mutant receptors might also be expected to clarify the role of ER or other estrogen-binding proteins in these processes (Monchamont *et al.*, 1985).

C. Alternative Mechanisms of Estrogen Action

In addition to classical hormone receptors originally observed in cytosolic extracts of target cells, other estrogen binding sites have been proposed on the basis of intracellular compartmentalization, differing kinetics or specificities in hormone binding, or such physico-chemical properties as density gradient profiles. Several investigative groups have identified high affinity binding sites for estradiol in various particulate cellular fractions; i.e. nuclear matrix (Barrack and Coffee, 1980); microsomes (Patrick *et al.* 1980; Watson and Muldoon, 1985); cytoskeleton (Sica *et al.*, 1985); plasma membranes (Pietras and Szego, 1979; Berthois *et al.*, 1983).

Some specific estrogen effects, such as long-term uterine growth in rodents (Markaverich and Clark, 1979), and the long-term induction of apolipoproteins E and B in human hepatocytes (Tam *et al.*, 1986; Hache *et al.*, 1987), appear to correlate best with the presence and the induction of so-called type II estrogen-binding sites ($K_D = 5x10^{-8}M$). In addition to these estradiol binding sites, a class of binding sites specific for non-steroidal anti-estrogens may exist (Sutherland *et al.*, 1980; Miller *et al.*, 1984). However these sites are not restricted to estrogen target tissues. In summary, the role of any of these alternative steroid binding sites in physiological responses to estradiol or antiestrogens is unknown.

Functionally steroid hormone action may not be limited to altering the transcription rate of specific genes. Steady state levels of mRNA can be affected by alteration in degradation rates as well as by change in transcriptional activity. Estradiol increases the stability of vitellogenin mRNA in *Xenopus* (Brock and Shapiro, 1987); conversely, estradiol decreases the stability of albumin mRNA (Riegel *et al.*, 1986). The ER may mediate the post-transcriptional regulation of albumin mRNA levels (Riegel *et al.*, 1987b). Prior administration of 4-hydroxy-tamoxifen blocks both the suppression of albumin mRNA and the activation of vitellogenin transcription. Other recent reports indicate that estradiol itself regulates ER mRNA levels via both post-transcriptional and transcriptional mechanisms (Saceda *et al.*, 1988).

It is also possible that steroid hormones could alter translation and post-translational processes in target tissues. Glucocorticoid administration alters the post-translational phosphorylation and glycosylation of MMTV proteins (Firestone *et al.*, 1982; Haffar *et al.*, 1987; Firestone *et al.*, 1986). Glucocorticoids inhibit the translation of five out of six ribosomal protein (rp) mRNAs as measured by a decrease in ribosomal loading (Meyuhas *et al.*, 1987). In the case of estradiol, the amount of an EBP (estrogen binding protein) associated with ribosomes is inversely correlated with the rate of peptide elongation on rat uterine ribosomes (Labate *et al.*, 1986). Estradiol also differerentially alters the glycosylation of uterine proteins, possibly by stimulation of mannosylphosphory-dolichol synthase mRNA synthesis (Dutt *et al.*, 1985; Carson *et al.*, 1990).

Steroid hormones thus can apparently influence gene expression at several functional levels. RNA transcription, nuclear processing, and cytoplasmic stabilization, as well as protein translation, localization and post-translational modification, represent potential targets for hormone action. To date, however, no study has confirmed that any proteins other than the classical steroid hormone receptors mediate these effects. The direct involvement of alternative binding sites in translation, post-translational processing and/or

secretion, peptide receptor internalization, microtubular dissociation and mitosis remains highly speculative.

IV. Regulation of Cell Growth

A. General Model - The Cell Cycle

Conventionally the cell cycle is divided into Gap 1 (G1), DNA synthesis (S), Gap 2 (G2), and mitosis (M). For most dividing cells the variability in generation time between different dividing cell lines, and within dividing cells of a single cell line, is attributed to variation in the length of G1 (reviewed by Prescott, 1976). The concept of checkpoints for cell arrest in G1 has been gradually developed from observations in cell lines stopped in G1 by density arrest, nutrient deprivation, or otherwise unfavorable environmental conditions (Campisi *et al.*, 1982; Pardee, 1974). G0 was originally introduced to distinguish between cells which have withdrawn from the cell cycle and cells that are transiently arrested, or progressing at some finite rate through G1.

A set of discrete functional components in serum is essential for cellular traverse of fibroblasts through G1 (Leof *et al.*, 1982; Smith and Stiles, 1981; reviewed by Stiles, 1985). One of these factors, (PDGF, absent in platelet-poor serum), is essential for an optimum mitogenic response, but is not itself sufficient. A second set of factors, present in platelet-poor plasma, are also necessary. Exposure to the 'competence' factor, PDGF, is only needed for a brief period during cell cyle progression. The second set of 'progression' factors (EGF, somatomedins) are required constantly.

A recent kinetic analysis of Swiss 3T3 cells concluded that transient inhibition of protein synthesis (due to serum deprivation or cycloheximide treatment) only produces cell cycle arrest if treatment occurs in the first three to four hours of G1 (Zetterberg and Larsson, 1985). This procedure forces most cells into a quiescent G0 state that lasts eight hours. PDGF alone, although not EGF or insulin, is capable of fully counteracting serum deprivation and completing the committment process. The G1 phase has therefore been divided into a 3.5 hour (on the average) serum-dependent post mitiotic period (G1pm), and a variable serum-independent, pre-DNA synthesis period (G1ps). New RNA synthesis is required during the G1pm period. Fusion of cytoplasts from cells briefly exposed to PDGF to quiescent, untreated Balb/3T3 cells produces heterokaryons capable of DNA replication on incubation in plasma-supplemented medium (Smith and Stiles, 1981).

If RNA synthesis is blocked in the PDGF-treated cells, transfer of the growth response is prevented. Exposure to cycloheximide does not prevent transfer of the mitogenic activity (Leof *et al.*, 1982).

The transcription rate of many specific genes is known to vary with the cell cycle; i.e. dihydrofolate reductase (Farnham and Schimke, 1986), thymidylate synthetase (Navalgund *et al.*, 1980), histones (Hereford *et al.*, 1979), etc. Recently positive regulatory sequences nearby, or within, the structural genes for thymidine kinase (Lewis and Matkovich, 1986; Kim *et al.*, 1988; Stewart and Conrad, 1987), dihydrofolate reductase (Gasser and Schimke, 1986), and yeast histones (Osley *et al.*, 1986; Wright and Bishop, 1989) have been identified. A serum response element (SRE) required for transient transcriptional activation following treatment with serum or other mitogenic agents (insulin, EGF, phorbols) is associated with various genes (Visvader *et al.*, 1988). In the proto-oncogene *fos*, two independent SRE promoter regions at -308 and -280 appear to mediate this response, and two distinct nucleoprotein complexes interact with these elements. The SRE has a basal constitutive activity. cDNA clones for a serum response factor (SRF) which binds to the SRE have also been isolated (Norman *et al.*, 1988). SRF binds as a dimer, appears to be a ubiquitous nuclear protein, and has a striking homology to two known transcriptional regulators in yeast. Similarly, negative elements which confer periodic transcription and cell cycle regulation have been partially characterized (Gudas *et al.*, 1988; Farnham and Schimke, 1986). One of the promoter regions involved in the cell cycle specific regulation of histone gene expression in yeast is a negative regulatory element that represses transcription at specific points in the cell cycle (Osley *et al.*, 1986). The question of negative control, and loss of a repressor function, as a prelude to cellular division thus remains a viable research concern.

The mechanisms governing cell cycle regulation of gene expression are not established. For example, dihydrofolate reductase (DHFR) mRNA levels are both transcriptionally and post-transcriptionally regulated during the serum-stimulated transition from a resting to a growing state (Kaufman and Sharp, 1983). Similarly post-transcriptional processing appears to be important in the cell cycle regulation of histone mRNAs (Luscher and Schumperli, 1987). A 180 nucleotide fragment derived from the 3'

end of exon 1 of c-*myc* reduces transcriptional elongation when placed within an intron of the globin gene and assayed by transfection into HeLa cells (Wright and Bishop, 1989). The authors propose that attenuation may regulate transcription rate; however, the mechanism of attenuation in eukaryotic systems is unknown.

B. Oncogenes and Anti-oncogenes

The second major concept in the evolving cell cycle model is that of a family of regulatory genes whose individual members are specific for regulation of cell reproduction in various differentiated cell types. The thought that specific gene products affect cellular proliferation gathered impetus from the identification of cellular proto-oncogenes and viral oncogenes (reviewed by Bishop, 1985). The function of these genes and the biochemical nature of their various products is not fully understood. One large group of proto-oncogenes appears to function either as growth factors [c-*sis*, PDGF B-chain (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983)], or as growth factor receptors [c-*erb*B-1, truncated EGF receptor (Downward *et al.*, 1984)]. It is currently thought that the transmembrane domain of such receptors can confer much of the specificity for a given extracellular signal. Although many of the plasma membrane receptors for such growth factors have an associated protein kinase activity, the means of coupling such membrane signals to specific intracellular responses again remains unclear.

A second class, for which the prototype is c-*ras*, is thought to belong to to the family of signal transducing G proteins (McCormick, 1989; Bishop, 1987). *Ras* is critical for G1 progression and initiation of chromosome replication. The third group of oncogene products are known to have protein kinase activity [i.e. c-*src*, (Bishop, 1987)]. In the case of both the *ras* and *src* families, the intermediary mechanisms altering gene expression and cellular phenotype (i.e. transformation) are unknown. However, a number of studies implicate protein kinase C and phosphatidyl-inositol turnover as possible events in signal transduction.

The fourth class of oncogenes encode proteins which appear to act as trans-acting activators or repressors of gene transcription, i.e. c-*myc* (Einat *et al.*, 1985). Very recently

the c-*jun* gene product has been specifically shown to function as a transcriptional regulator (Bohman *et al.*, 1987; Struhle, 1988). This last class of genes is thought to function in the regulation, not only of cell growth, but of cellular differentiation. The latter conclusion is supported by the identification of the oncogene *int*-1 as a segment polarity gene controlling morphogenesis in Drosophila (Rijsewijk *et al.*, 1987). An association between the the relatively highly characterized family of transcriptional regulators represented by steroid receptors and the oncogenes subsequently developed when c-*erb*-A was reported to encode a thyroid hormone receptor (Weinberger *et al.*, 1986). Thyroid hormones are known to influence the development of many tissues, notably the nervous system. The identification of TR as the proto-oncogene c-*erb*-A has thus provided a direct link between the nuclear binding trans-acting hormone receptors and processes resulting in oncogenic transformation (Green and Chambon, 1986). Similarly the morphogen retinoic acid interacts with its steroid family receptor (RAR), functioning in the growth and maintenance of epithelial tissue and inducing differentiation in a number of cell types *in vitro* (Petkovich *et al.*, 1987). Specific mRNAs appear on differentiation of F9 teratocarcinoma cells into partial endoderm following treatment with retinoic acid (La Rosa and Gudas, 1988).

Considerable evidence links proto-oncogene expression and cell cycle progression. Cellular *ras* gene expression increases during middle to late G1 in many cultured cells, and is essential in G2 (Durkin and Whitfield, 1987). Agents such as PDGF alter the expression of some oncogenes (Liboi *et al.*, 1986), as well as a variety of other specific mRNAs and peptides (Cochran *et al.*, 1983; reviewed by Heldin and Westermark, 1984). C-*fos* and several other cell cycle genes respond to PDGF only when cells are in G0 and confluent; in contrast c-*myc* expression is maximally induced by PDGF in cycling cultures (Rollins *et al.*, 1987). NGF induces proto-oncogene *fos* expression in rat pheochromocytoma cells and two promoter elements (SREs) appear to mediate this response (Visvader *et al.*, 1988). c-*jun* is undetectable in quiescent mouse fibroblasts, but is serum-inducible during the G0-G1 transition (Ryseck *et al.*, 1985; Ryder *et al.*, 1988). A clone (AH119) hybridizing to two mRNA species of 2.3 and 2.7 kb has since been isolated by differential screening of quiescent vs. cycling mouse fibroblasts (Ryseck *et al.*, 1988). This clone has a mRNA half-life of 15 minutes and is the mouse homologue of c-*jun*.

Oncogene products (Fos, Myc, Jun, Ras) may both regulate, and be regulated, by other cell-cycle specific proteins (Sassone-Corsi and Verma, 1987). Two proteins (42 and 45-KDa) which share an antigenic epitope with the DNA-binding domain of c-*jun*, but which diverge at the amino terminus have recently been reported (Sharma *et al.*, 1989). These proteins interact specifically with a 32 nucleotide sequence which confers cell cycle regulation of the H3.2 histone gene. The authors suggest that members of the *jun* family may play specific roles in the expression of genes essential for cell cycle progression.

The oncogenes are generally viewed as positive effectors in the regulation of cell division. However there is considerable evidence that similar gene products may function in the negative control of cell reproduction. This theory was originally based on genetic evidence obtained in *Xiphophorus* crosses (Schwab and Scholl, 1981), and on epidemiological data [retinoblastoma (Comings, 1973; Murphree and Benedict, 1984); Wilm's tumor (Knudsen and Strong, 1972)]. Somatic cell hybridization studies have often suggested that normal cells posess tumor suppression mechanisms (reviewed by Sager, 1985). Such negative regulatory mechanisms have been attributed to effects of the gene products of so-called anti-oncogenes.

The recently cloned and sequenced retinoblastoma (Rb) gene provides the most dramatic example of an anti-oncogene. The neoplastic phenotype of retinoblastoma, prostate cancer, or osteosarcoma can be suppressed by introducing an exogenous Rb gene into cells which have inactivated endogenous Rb genes, thus confirming a direct role of Rb in tumorigenesis (Huang *et al.*, 1988; Bookstein *et al.*, 1990). The mechanism of action is unknown. It has recently been reported that retinoblastoma cells, in contrast to fetal retinal cells, lack TGF-β receptors and fail to respond to this growth-inhibitory agent (Kimchi *et al.*, 1988). However, the Rb gene product itself is believed to function as a dimeric DNA binding protein and transcriptional regulator. The Rb monomer is capable of interacting directly with, and modifying the function of other transcriptional regulatory proteins. For example, SV40 transformation may involve either perturbation of either Rb protein function or of T antigen function through complex formation between the two proteins (DeCaprio *et al.*, 1988). The phosphorylation state of Rb protein varies with the cell cycle (unphosphoryulated in G1, phosphorylated in S/G2); only unphosphorylated Rb

complexes with T antigen (Ludlow *et al.*, 1990). Similar complexes of Rb gene product with the E7 protein of human papillomavirus and adenovirus E1A have also been reported. A nonfunctional Rb gene detected in J82 bladder carcinoma cells is mutated only in the region of the protein essential for protein complex formation; DNA binding is unaltered (Horowitz *et al.*, 1989).

Other tumor suppressor genes may function differently. One, known as Krev-1, encodes a protein with homology to Ras and blocks transformation by the viral Ki-*ras* oncogene (Kitayama *et al.*, 1989; Noda *et al.*, 1989). Krev-1 is ubiquitously expressed in many rat organs and a variety of eukaryotic species. This cDNA clone is derived from one of seven revertants with resistance to activated *ras*. A second suppressor gene appears to function in regulating the activity of a secreted inhibitor of angiogenesis (Rastinejad *et al.*, 1989). P53, originally thought to be an indicator of transformation, is now in fact believed to function as an anti-oncogene (Dippold *et al.*, 1981; Green, 1989). The transforming potential of p53 is activated by a wide variety of mutations, suggesting that inactivation of this protein is a critical element in phenotypic alterations. Like Rb, p53 forms multimers and interacts with SV40 large T antigen (albeit with a different region of that protein). Other discrete genetic loci associated with the development of specific malignancies may also have been identified; i.e. Wilms tumor gene (Gessler *et al.*, 1990); melanoma on human chromosome 6 (Trent *et al.*, 1990); ademomatous polyposis coli on chromosome 5 (Solomon, 1990).

A variety of mechanisms of regulation of oncogene expression have been reported. Changes in steady-state levels of *myc* RNA are attributed to change in both rate of transcription and of stability of mRNA (Einat *et al.*, 1985). C-*myc* expression in growth arrest of F9 teratocarcinoma cells is thought to be post-transcriptionally regulated (Dean *et al.*, 1986). Jun/AP-1 activity in phorbol-stimulated cells is regulated by transcriptional activation of *jun* gene expression and by unknown post-translational modifications which increase DNA binding activity (Angel *et al.*, 1988).

A particularly complex and interesting picture has emerged from the study of the transcriptional activator Jun/AP1. Jun/AP1 appears to regulate the expression of a number of other genes, including c-*fos*, and c-*jun* itself. In contrast to c-*myc* and c-*fos* gene

products which negatively autoregulate the transcription of their cognate genes, Jun/AP-1 activates c-*jun* gene expression by binding to a TPA recognition element (TRE) in the 5' flanking region (Angel *et al.*, 1988). Jun/AP-1 binds to DNA as a dimer, and like the Rb gene product, can interact with other proteins (Turner and Tjian, 1989); a "leucine zipper" motif is thought to mediate the oligomerization of these proteins (Dong *et al.*, 1989; Scheurmann *et al.*, 1989). It has similarly been shown that Fos and Jun can interact to form a heterodimeric TRE-binding complex (Gentz *et al.*, 1989; Halazonetis *et al.*, 1988). The Fos-Jun complex is even more effective than the Jun homodimer in stimulating c-jun transcription; however, the Fos-Jun dimer apparently also rapidly down-regulates c-*fos* transcription after serum stimulation (Angel *et al.*, 1988). The rate of transcription of genes regulated by such dimeric proteins [Fos, Myc, Jun (*Dang et al.*, 1989)] clearly may depend upon the concentrations and relative affinities of these proteins for homo- or heterodimeric protein complex formation. It is also obvious that a plethora of possible complexes with similar DNA binding proteins could exist, and each could manifest a distinct pattern of gene activation or repression (Nakageppu *et al.*, 1988). Overexpression, underexpression, or mutation of a single monomeric species could thus produce aberrant transcription complexes which cause pleiotropic changes in gene expression. Further, such phenotypic phenomena as transformation could be attributed to the functional inactivation of a gene product which normally suppresses the activity of another regulatory protein via complex formation (Myc, Rb). Such mutations would be negative dominant in trans.

Amplification or rearrrangement of several protooncogenes (*myc, neu*) has been thought to contribute to the formation and progression of some human malignancies. However there is considerable controversy concerning the role of proto-oncogenes in human breast cancer, and this specific issue will be further addressed in a later discussion of mammary cancer. The function of most of these oncogenes is unclear; the mechanisms by which such events as transformation are influenced, including the functionally specific gene products which are oncogene targets, is unknown; and the molecular mechanisms regulating the expression of these oncogenes appears to be extremely complex (reviewed by Klein, 1987).

C. Evidence that Specific Genes Regulate Cell Division

Formal evidence that unique copy gene transcription is necessary for transition from a quiescent to a growing state (G1-S) has remained elusive. Cell cycle mutants have been identified in yeast (Nurse, 1985), as well as mammalian cell lines [chiefly in chinese hamster cells (Marcus *et al.*, 1985)]. To date the only firm data on possible genetic loci have been obtained with the CDC (cell division cycle) genes in yeast (Nurse, 1985). Computer based sequence analysis of the cdc genes identifies a repeating 34 amino acid motif present in three of these genes and in two additional genes regulating RNA synthesis. These are collectively termed the TPR family (Sikorski *et al.*, 1990). At least one of these genes (CDC2+) in fission yeast codes for P34, a protein kinase (Simanis and Nurse, 1986). In budding yeast, a functionally equivalent gene (cdc28) has also been identified, and cdc28 has a human homologue of similar sequence (Draetta and Beach, 1988; Lee and Nurse, 1987). The most highly conserved regions of all three gene products are associated with kinase activity. Loss of cdc28 function by mutation prevents progression from G1 to S phase (reviewed by Solomon *et al.*, 1988). Also P34 is dephosphorylated and loses kinase activity when cells cease to proliferate and arrest in G1. In addition to its regulatory role in G1 transition, P34 is also a component of an M-phase specific histone H1 kinase; the latter kinase complex is thought to be identical with maturation promotion factor (MPF) and represents a critical element in regulating the second cellular transition from interphase to metaphase (Arion *et al.*, 1988). $P34^{cdc28}$ is thus a polypeptide component of a protein complex which phosphorylates a number of different substrates; the assembly and disassembly of this complex is associated with kinase activity and control of the yeast cell cycle (Draetta and Beach, 1988; Wittenberg and Reed, 1988).

A group of functionally redundant molecules called cyclins are also essential to cell cycle progression through M phase in higher organisms ; these are homologous to the cell cycle gene cdc13 in yeast, and their expression characteristically increases during interphase and drops during exit from mitosis (Almendral *et al.*, 1987). It has been suggested that cyclin-like products also interact with MPF (Solomon *et al.*, 1988); indeed, clam cyclins A and B are now thought to be components of the MPF complex (Gautier *et*

al., 1990). The selective proteolysis of these molecules is believed to provide the mechanism of MPF inactivation and exit from mitosis (Draetta *et al.*, 1989). Cyclins again have a dual role in cell cycle progression since they also appear to activate P34 and are thus essential for the G1-S transition (Richardson *et al.*, 1989). Still another protein component of MPF has now been cloned and identified as PCNA (proliferating cell nuclear antigen); this DNA binding protein is in fact an auxiliary protein for DNA polymerase (Almendral *et al.*, 1987; Jaskulski *et al.*, 1988).

In addition to genetic evidence for regulation of cell cycle progression, the presence or absence of a number of gene products appears to be phase-specific. Two dimensional gel electrophoresis of proteins produced during various cell cycle phases reveals a number of discrete species (Thomas *et al.*, 1981). Four new proteins, two of which are transiently expressed, have been identified by 2D gel electrophoresis following serum stimulation of 3T3 cells. Actinomycin D prevents the accumulation of several of these, including the transiently expressed N26 and N47. A protein termed "dividin" is first seen in transformed human amnion cells late in G1 phase and is maximal during late S phase. This M_r 54,000 protein is not detected in growth arrested epithelial or fibroblast cells (Celis and Nielson, 1986). Similarly a protein designated as p27 is induced by serum treatment of growth-arrested 3T3 cells (Santaren and Bravo, 1986).

Appearance of other specific cellular proteins is associated with particular growth states: i.e. a M_r 57,000 protein termed statin is seen only in senescent and quiescent cells (Wang, 1985; Ching and Wang, 1988). Three additional proteins disappear from medium when human fibroblasts reach senescence, and at that time a M_r 5,000 protein appears. It should be noted that the senescent growth state is distinct from quiescence. Senescent cells cannot be stimulated to enter the S phase of the cell cycle by any combination of growth factors or physiological mitogens (Seshadri and Campisi, 1990). Further, cell hybridization results suggest that one or more dominant genes localized on chromosome 1q are critical genetic elements in regulating senescence (Sugawara *et al.*, 1990).

It is likely that the cell cycle dependent appearance or disappearance of specific proteins reflects alteration in levels of specific mRNAs. Temperature-sensitive mutants in RNA polymerase II (TsAF8 BHK cells) arrest in G1 at the non-permissive temperature

(Baserga, 1984). R_0t analysis performed by cross-hybridizing cDNA and mRNA from resting and exponentially growing mouse 3T6 cells indicates that some 3% of the sequences from either population do not hybridize (Linzer and Nathans, 1983). These observations collectively suggest that specific changes in gene expression do indeed occur during the cell cycle, and in the transition from a quiescent to a growing state.

D. Cloning of Growth-Regulatory Genes

Several groups have prepared cDNA libraries from dividing and quiescent cell lines, and then used a strategy of differential screening to isolate specific genes. Some of the most interesting and productive experimental systems are discussed below. Screening a cDNA library from serum-stimulated Balb/3T3 cells with cDNA from either stimulated or quiescent cells identified thirteen clones differentially expressed in growing cells (Lau and Nathans, 1985; Lau and Nathans, 1987; Linzer and Nathans, 1983). One clone, proliferin, is maximally expressed during late G1; regulation appears to be post-transcriptionally mediated (Linzer and Wilder, 1987). Nine of the other clones appear to be immediate early genes co-ordinately regulated with c-*fos* and c-*myc* (Lau and Nathans, 1985). Poly(A) RNA corresponding to some of these clones fluctuates over a twenty fold range in a manner consistent with cell growth patterns; PDGF and 20% serum stimulate mRNA accumulation dramatically. At least one clone encodes a protein with zinc finger sequences which appears to be a member of the steroid and thyroid homone receptor family (Hazel *et al.*, 1988; Christy *et al.*, 1988.

A similar strategy has been used in Balb/3T3 cells treated with PDGF to isolate fifty-five clones hybridizing only with the cDNA from PDGF-treated and cycling cells (Cochran *et al.*, 1983). Forty-six of these clones represent five independent gene sequences. Nine inserts are too small to analyze. Limits of detection during the screening process are calculated as 500 copies per cell. Further analysis of two of these clones indicated that 3000 and 700 mRNA copies respectively are present in PDGF-treated cells, and 100 and 70, copies respectively are present in quiescent cells. One of these genes, JE, encodes a product homologous to interferons α and $\beta 2$, and to interleukin 2 (Rollins *et al.*,

1988). mRNA levels for JE, as well as clones KC and JB, are induced one hour after PDGF treatment; other growth factors, which do not bind to PDGF receptors, fail to stimulate mRNA levels (Rollins *et al.*, 1987). It should be noted that under these experimental conditions PDGF-treated cells remain growth-arrested, since no progression factors are present.

Still another study utilized differential hybridization with radiolabeled cDNA prepared from G0 and G1 cell populations to screen a cDNA library from a G1-specific cell cycle mutant (ts13) derived from Syrian hamster BHK cells. The expression of approximately five hundred clones increased at various points in G1 transit (Hirschhorn *et al.*, 1984). No unique clones have been identified, possibly because 10% of cells ostensibly in G0 are actually cycling. The ts13 mutation (temperature-sensitive mutant in RNA polymerase II) was then used to distinguish early G1 (G1pm) expression from late induction (G1ps). Three of the G1pm clones have homology with known oncogenes.

Baserga and colleagues have cloned a cDNA for a growth factor inducible gene with strong homology to S-100, a calcium-binding protein (Calabretta *et al.*, 1986). This clone, 2A9, increases in response to PDGF and EGF with maximum induction in G1 phase. The gene is characteristically amplified and mRNA levels are elevated in myeloid leukemia. This same research group has also identified a number of other cell cycle regulated clones. 2A9, 2F1, and 4F1 increase approximately four hours after serum stimulation in WI-38 fibroblasts and decline after twenty-four hours (Rittling *et al.*, 1986). Unfortunately, no difference in expression is seen between the effect of serum on young fibroblasts induced to replicate and on senescent non-replicating fibroblasts. The clone pJE-3 originally isolated by Stiles and collaborators (described above) shows a similar pattern of expression (Rollins *et al.*, 1988).

Two genes (Krox 20 and Krox 24) which demonstrate transient activation by serum growth factors have been cloned from a mouse 3T3 cell library in λNH1149 on the basis of cross-hybridization with the zinc finger encoding region of Kruppel (LeMaire *et al.*, 1988; Almendral *et al.*, 1988). The temporal pattern of activation resembles that of c-*fos*; mRNAs are expressed at the G0-G1 transition and display tissue specific expression patterns (Le Maire *et al.*, 1988). A weak SRE in the 5' flanking region and a strong SRE

in the first exon of Krox 20 have been identified; this element can functionally substitute for the SRE of c-*fos* and binds the SRF. Cycloheximide treatment superinduces these genes, due to both mRNA stabilization and prolongation of the period of transcriptional activation (Chavrier *et al.*, 1988; Chavrier *et al.*, 1989). Both Krox 20 and 24 encode proteins containing three zinc finger domains. Consistent with the link between processes governing cell growth and cellular differentiation, Krox 20 demonstrates segmental and temporal patterns of expression during the development of the mouse nervous system (Wilkinson *et al.*, 1989).

A few other isolated examples of cloned genes associated with cell cycle regulation, cell growth, and/or differentiation are summarized below. Human (EGR-2) and mouse (EGR-1) early growth response genes which are responsive to both fibroblast and lymphocyte mitogens, and which are homologous in the zinc finger region to the cys2-his2 subclass of DNA binding proteins, have been partially characterized (Joseph *et al.*,1988). A cDNA, ts11, which is induced in mid G1, and is itself essential for progression through G1has been cloned (Greco *et al.*, 1987). La Rosa and Gudas (1988) have isolated a 2.2-2.4 kb cDNA which is rapidly induced during teratocarcinoma stem cell differentiation. Similarly Iffman *et al.*(1987) have identified a 2 kb cDNA clone (BN51) which permits progression through a G1 block in BHK cells. Calcyclin, a member of a gene family including cystic fibrosis antigen and calpactin I, is also responsive to various growth factors (Jackson-Grusby *et al.*, 1987; Gezzo *et al.*, 1988).

One research group has proposed that isolating possible negative regulators of cell division is feasible. Microinjection of poly(A) RNA from quiescent and senescent fibroblasts inhibits DNA synthesis in proliferating HeLa or diploid fibroblast cells (Pepperkok *et al.*, 1988); a particular high abundance RNA (0.8% of total mRNA) is implicated in this effect (Lumpkin *et al.*, 1986). A subtraction library from RNA enriched for sequences preferentially expressed in growth-arrested BNIH 3T3 cells has been prepared (Schneider *et al.*, 1988). Six distinct clones, representing 2% to .002% of the library sequences, have been identified and designated gas genes. The corresponding mRNAs downregulate with varying kinetics on serum induction of growth.

The above evidence suggests that specific gene transcription is associated with cell cycle progression and with the regulation of cell growth. Several oncogenes, and other genes which may have a regulatory function, have already been isolated using a differential screening strategy. However, additional biological models would facilitate investigations into mechanisms of growth regulation. Previous discussion has indicated that estradiol functions as a trophic hormone in a number of tissues; one such system, the hormonally responsive MCF-7 cell line, will be discussed below.

V. Effect of Estradiol on MCF-7 Cells

A. Estrogen and Estrogen Receptor in Mammary Cancer

Empirical observations concerning the relationship between the genesis of mammary cancers and reproductive status date back to 1713 (Ramazzini, 1964). Epidemiological information, i.e. the relationship between nulliparity and increased risk of breast cancer, accumulated throughout the 19^{th} century (Cooper, 1835). The first experimental evidence for hormonal dependency of some breast cancers was obtained when ablative treatment (ovariectomy) was employed as a therapeutic measure (Beatson, 1896) . Despite continued empirical manipulation of the endocrine system, a mechanistic explanation of these clinical observations awaited the identification of specific estrogen receptors in estrogen responsive tissues. Jensen (1967) first reported that tumors from patients who responded to hormonal therapy contained ER in the cytoplasm, while those that are unresponsive lacked receptor.

In 1974, a workshop conducted by the Breast Cancer Task Force evaluated data from fourteen different institutions (McGuire *et al.*, 1975. This study concluded that patients whose breast cancers lacked a significant quantity of ER (<10 fmol/mg protein) had little chance of response to hormonal manipulation; however, approximately seventy percent of patients classified as ER positive would benefit from such therapy. This workshop evaluated both the methodologies and actual data, and established the criteria for classification of tumors as ER+ and ER-. Subsequent investigations have confirmed the prognostic value of ER determinations in endocrine therapy of breast cancer. Presence of PR (progesterone receptor), an estrogen-induced protein, is also often used as a predictive marker (Horwitz and McGuire, 1978).

B. Mammary Models for Estrogen Action

The mammary gland presented a difficult model in which to delineate the mechanism of estrogen action. Clinical and epidemiological evidence indicated that estradiol influences the growth of mammary tumors (reviewed by Seibert and Lippman, 1982). Histological and biochemical studies similarly suggested a functional role for estradiol in the growth, morphology, and intermediary metabolism of the normal organ (reviewed by Cowie and Folley, 1961; Lyons *et al.*, 1958). However, the hormonal regulation of mammary tissue is extremely complex. All classes of steroids (estrogens, progestins, glucocorticoids, androgens, mineralocorticoids), pituitary hormones (prolactin, growth hormone, oxytocin), as well as thyroxine and insulin, appear necessary for normal differentiation, growth and lactation (Segaloff, 1966; Topper and Freeman, 1980). Additionally mammary tissue is comprised of a spectrum of cell types (ductal epithelium, lobular-alveolar epithelium, myoepithelium, various components of connective tissue) with apparently distinct responses to hormone treatment (Dulbecco *et al.*, 1986; reviewed by Topper and Freeman, 1980). For example, tissue interactions in mammary gland involve both epithelial-epithelial and stromal-epithelial cell types; the effect of androgens on the embryonic development of mouse mammary gland anlagen is mediated by mesenchyme (Durnberg *et al.*, 1978). Dissection of the hormonal mechanism of regulation clearly required experimental models to isolate effects of estradiol. Further, some investigators questioned whether the mechanism of action of estradiol applied to both normal and tumor tissue.

Rat mammary tumors provided the first such model systems for mammary cancers. Methycholanthrene induces tumors in female Sprague-Dawley rats, and approximately 95% of those tumors classified as adenocarcinomas regress following ovariectomy (Huggins *et al.*, 1959). Subsequently three lines of transplantable tumors, a spontaneous fibroadenoma in Sprague-Dawley rats (Dunning, 1960), the DMBA-induced tumor line 13762 in a specific strain of Fischer rats (Segaloff, 1966), and a carcinoma line termed R3230 in Fischer rats (Hilf *et al.*, 1964) were established. In general, the growth rate of these tumors increases in response to estradiol, and growth is adversely affected by

antiestrogens. The results of experimental hormonal manipulation correlates highly with the results of similar clinical treatment of human mammary cancer (Simpson-Herren and Griswald, 1970; reviewed by Hilf *et al.*, 1967). Further the general biochemistry and the pattern of response to prolactin, insulin etc. are very similar in tumor and normal rat mammary tissue (reviewed by Welsch, 1982).

One major consequence of these studies has been exension of the mechanistic information derived from normal tissue to tissue that is clearly neoplastic. It seemed probable that the basic mechanism of estrogen action, i.e. modulation of transcription via a specific intracellular mediator (ER), in normal tissue, applied to both normal and mammary tumors. However the question of whether estradiol directly stimulated tumor cell growth could not be easily resolved in an *in vivo* system. Additionally, since species differences in mammary tissue constituted an experimental issue, a human model for mammary cancer appeared very desirable (Hilf *et al.*, 1967).

Organ cultures of human mammary tissue (both normal and neoplastic) do permit evaluation of direct effects of hormones on tissue. Expression of differentiated functions often requires a precise architectural organization, as well as interaction among varied cellular components of a given tissue (Dulbeccho *et al.*, 1986; Simpson-Herren and Griswald, 1970; Suard *et al.*, 1983). Although these systems offer the advantage of mimicking parent tissue organization, limited tissue availability and limited viability effectively restrict use of such organ models.

In 1973, Soule and coworkers established a human breast cancer cell line designated as MCF-7 (Soule *et al.*, 1973). This line is derived from a pleural effusion of a post-menopausal patient with metastatic breast cancer. The cell line has met numerous critera of morphological, biochemical, and functional differentiation which confirm a human mammary epithelial identity. The histological organization of monolayer cultures reflects their origin as ductal epithelium (Pourreau-Schneider *et al.*, 1984; Russo *et al.*, 1977). Cells demonstrate acinar-like organization and lumen formation with characteristic secretory epithelial morphology, including well developed endoplasmic reticulum and Golgi apparatus. Junctional complexes and microvilli are evident at the luminal surfaces. Cellular responses to a variety of hormones and other biological agents, as well as specific

cellular receptors to those agents, have been demonstrated (Horwitz *et al.*, 1975; reviewed by Kasid *et al.*, 1985). MCF-7 cells possess high levels (30-100 fmol/mg protein) of ER; this observation is significant in view of the imposing body of clinical information on the diagnostic value of ER in human mammary cancer.

Long-term tissue culture offers many advantages in that the population is relatively homogeneous, large quantities of cells are continually available, and precise, stringent control of experimental conditions can be achieved. Thus experimental variability tends to be reduced. However, the loss of expression of some differentiated functions is a negative aspect of cell growth in monolayer culture; for example MCF-7 cells do not produce casein (Monaco *et al.*, 1977), and synthesis of α-lactalbumin is low and not hormonally regulated (Rose and McGrath, 1975). The genetic instability of such malignant cell lines also presents a recurring problem (Seibert *et al.*, 1983). MCF-7 cells have been distributed to a number of different laboratories, and different sublines appear to have evolved from the original uncloned heteroploid line (Briand and Lykkesfeldt, 1984; Darbre *et al.*, 1983).

Over the past 12 years, MCF-7 cells have become the major *in vitro* model for investigations into 1. the biochemical nature of ER and ER cellular dynamics; 2. the design and functional assay of new antiestrogens; 3. the molecular mechanisms by which estradiol modulates both cell growth and specific differentiated functions; 4. development of hormonal and chemotherapeutic protocols for treatment of human breast cancer; 5. investigations into the mechanisms of multi-drug resistance. Although a number of other human mammary cancer cell line have since been established (Yu *et al.*, 1981; Horwitz *et al.*, 1978; Westley *et al.*, 1989; reviewed by Engel and Young, 1978), the MCF-7 cell line has been most thoroughly investigated.

C. Effect of Estradiol on Growth of MCF-7 Cells

Estradiol stimulates the cell growth of MCF-7 cells as measured by increases in cell number, and in protein, RNA and DNA synthesis. Nonetheless, the past literature does include contradictory reports concerning the trophic effects of estradiol on MCF-7 cells (Briand and Lykkesfeldt, 1984; Darbre, 1983). In addition to possible variations among

sublines in different laboratories, variation in growth conditions is a significant factor in this controversy (Page *et al.*, 1983). It has been proposed that hormones and transport proteins can substitute for serum in supporting cell viability in culture (Richter *et al.*, 1972; Barnes and Sato, 1980). Therefore one approach to the control of growth conditions has been the development of defined media which will maintain cell growth in the absence of serum (Eagle, 1955); MCF-7 cells require medium supplementation with triiodothyronine and transferrin (Barnes and Sato, 1979; Lippman *et al.*, 1986). A second approach has been to completely remove endogenous estradiol from serum. Most cells in tissue culture can concentrate steroid hormones ten fold over medium by a non-receptor mediated process (Strobl and Lippman, 1979), and periods of up to two weeks may be necessary to deplete MCF-7 cells in culture of initial physiological levels of estradiol. Even low levels of serum supplementation with fetal calf serum or calf serum can expose cells to 10^{-10}M estradiol or estrogen conjugates (Esber *et al.*, 1973; Vignon *et al.*, 1983). Thus treatment of serum with sulfatase, as well as treatment with dextran-coated charcoal, is necessary to remove endogenous estradiol. On the other hand, prolonged deprivation of estradiol can result in the clonal selection of hormone-independent variants (Seibert *et al.*, 1983).

Under tissue culture conditions which completely strip serum of endogenous estrogens (Darbre *et al.*, 1985) and in defined medium (Lippman *et al.*, 1986), estradiol consistently increases cell number and the synthesis of DNA, RNA and protein. However, untreated cells do maintain a low level of growth and mitotic activity. The fact that estradiol is an absolute requirement to support the tumor growth of MCF-7 innocula into nude mice *in vivo* consequently created serious doubts concerning the legitimacy of *in vitro* models for hormone-dependent breast cancer (Shafie, 1980). A number of investigators questioned the direct role of estradiol as a mitogen, and proposed that a class of molecules termed 'estromedins' acted in a paracrine fashion to regulate mammary growth (Sirbasku, 1978). For example Sonnenschein and Soto (1980) postulated that estradiol caused the secretion of putative growth factors by such organs as liver; these 'estromedins' then acted specifically on estrogen target organs. In contrast, McGrath and colleagues have shown the estradiol need not enter the systemic circulation in the nude mouse to promote MCF-7 tumorigenesis; elevation of local estrogen concentration

promotes local but not distant tumor growth (Huseby *et al.*, 1984).

Katzenellenbogen and colleagues may have clarified this issue in a recent report. Their data suggest that the indicator dye phenol red, which is a standard component of tissue culture media, functions as a weak estrogen (Berthois *et al.*, 1986). This compound competitively displaces estradiol from estrogen receptor, stimulates the growth of ER+ MCF-7 cells, but not ER- MDA-MB-231 cells, and increases PR levels in MCF-7 cells. The antiestrogens tamoxifen and 4-hydroxy-tamoxifen inhibit MCF-7 cell growth only in the presence of phenol red. No effects of antiestrogens are seen in medium lacking indicator although an additional increment in PR levels and in cell growth is detected on treatment with estradiol. Other groups have since confirmed that, in the absence of phenol red, MCF-7 cells are dependent on exogenous estradiol for cell growth (Glover *et al.*, 1988; Welshons *et al.*, 1988). Significant variables determining estrogen responsiveness thus include presence or absence of phenol red, choice of medium and serum supplementation, time in culture, cell density, and steroid memory effects (Glover *et al.*, 1988).

Estradiol and antiestrogens primarily affect MCF-7 traverse of the G1 phase of the cell cycle (Weichselbaum *et al.*, 1978; Brunner *et al.*, 1989). Tamoxifen is a cell cycle phase-specific growth inhibitor and cytotoxic agent which decreases the percent of cells in S-phase and increases the proportion of G0-G1 cells (Sutherland *et al.*, 1983; Reddell and Sutherland, 1987). The effects of tamoxifen are exerted primarily during a short interval in mid-G1. In contrast, estrogen treatment increases the number of cells in S, G2 and M and decreases the proportion of cells in G0-G1. Unfortunately, all attempts to synchronize the growth of MCF-7 cells have been of limited success. Following growth arrest in early G1 due to isoleucine deprivation (Jakesz *et al.*, 1984) or antiestrogen treatment (Osborne *et al.*, 1983), cells enter S after a 12 to 24 hour delay as an asynchronous wave extending over a 12 hour time span. It is therefore unknown whether estrogens and antiestrogens act at specific points in the cell cycle by altering the expression of so-called 'competency' and 'progression' factors in G1, or by other mechanisms involving synthesis of specific gene products.

Evidence has accumulated that the growth of many tissues is influenced by both paracrine (Ikeda and Sirbasku, 1984) and autocrine (Sporn and Todaro, 1986) mechanisms. Factors derived from pituitary tissue (Cosby *et al.*, 1987), and from kidney, liver, and uterus (Sirbasku, 1978), stimulate the growth of mammary tumor cells. In addition, past studies have frequently suggested that cells in tissue culture 'condition' the surrounding medium, supposedly by secretion of metabolic intermediates or growth factors which then influence cellular proliferation (Puck *et al.*, 1956). Estradiol stimulates the production of a variety of secreted products by ER+ mammary tumor cells (Vignon *et al.*, 1983); conditioned medium from estrogen-treated MCF-7 cells increases while medium from antiestrogen-treated cells decreases the growth of these cells and other breast cancer cell lines. In addition, conditioned medium can at least partially substitute for estradiol in supporting tumor formation *in vivo* (Dickson *et al.*, 1986).

In many cell systems phorbol esters mimic the effect of specific serum growth factors, although the mechanism of such action is unknown. In contrast, TPA treatment of MCF-7 cells results in cell growth arrest, with a cell cycle blockade in G1 as well as delayed passage through G2 (Valette *et al.*, 1987). TPA also prevents the increased number of cells in S phase normally seen following administration of estradiol to MCF-7 cells which have been pretreated and synchronized with antiestrogens. Morphologically, phorbols increase cell volume as well as the number of ER secretory granules, mitochondria and microvilli.

MCF-7 cells secrete several specific growth factor activities (reviewed by Dickson *et al.*, 1987a), including FGF, TGF-α, TGF-β, IGF-1 related factor, an epithelial transforming factor, and PDGF. Estradiol stimulates, and antiestrogens inhibit, secretion of the IGF-1 related factor although an effect of estradiol on the secretion of IGF-1 is only seen when phenol red is removed from the culture medium and when the basal rate of secretion is lowered by growing cells in heat-treated, charcoal-stripped serum. A monoclonal antibody to IGF-1 receptor (Ab Alpha IR-3) blocks proliferation in the presence or absence of estradiol; however, estrogen-treated cells still maintain a higher level of proliferative activity than controls (Rohlis *et al.*, 1987). Additionally a product which biochemically is very similar to TGF-α, albeit of higher molecular weight, and which

apparently binds to EGF receptors, is induced by estradiol (Dickson *et al.*, 1985; Dickson *et al.*, 1986). Both IGF-1 and TGF-α are capable of replacing estradiol in stimulating MCF-7 colony formation in soft agar assays. PDGF-like activity, as measured by mitogenic response of Swiss 3T3 cells, is increased in medium conditioned by estrogen-treated MCF-7 cells approximately 3-fold (Bronzert *et al.*, 1987). Estrogens and insulin decrease, and antiestrogens increase secreted levels of TGF-β although neither intracellular levels nor mRNA levels are altered (Knabbe *et al.*, 1987). TGF-β inhibits the growth of these cells. In the antiestrogen resistant MCF-7 variant LY2, antiestrogens do not significantly increase TGF-β secretion; however, TGF-β does inhibit the growth of these cells.

The role of putative proto-oncogenes in cell growth has been considered previously. Some of the growth factors discussed above represent the products of oncogenes (PDGF), or bind specifically to proto-oncogene products (EGF receptors). A large number of studies have dealt with the role of oncogenes and anti-oncogenes in the genesis of mammary tumors, in the progression of tumors toward the malignant phenotype, and in the loss of hormonal dependency of such tumors. It is certainly clear that a genetic component contributes to the development of human mammary cancer. Genetic evidence implicates a highly penetrant autosomal dominant susceptibility allele for breast cancer in high risk families as well as the general population (Newman *et al.*, 1988). The nature of such a gene is unknown. It is also unknown, since breast cancer can be described as etiologically heterogeneous, whether genetic heterogeneity is due to different loci influencing susceptibility in various families, or whether different alleles at the same locus influence susceptibility. With respect to specific oncogenes the following observations have been recorded:

1. C-*erb*B-2 (also known as *neu*) tends to be amplified in aggressive human breast cancers with a poor prognosis (Van de Vijveret *et al.*, 1987; Slamon and Clark, 1987). In general amplification of the proto-oncogene *neu* results in overexpression of a gene product related to EGFR, and increases both the frequency of transformation of fibroblast cell lines *in vitro* and tumorigenicity *in vivo* (Hudziak, 1987; DiFiore, 1987). Leder and colleagues recently reported that the activated c-*neu* oncogene suffices to induce mammary

carcinoma in transgenic mice; in this study *neu* expression is driven by the MMTV promoter and the critical event may have been the activation of an oncogene at a specific temporal period in differentiation (Muller *et al.*, 1988). This group essentially concluded that the combination of tissue context and activation of an oncogene are major determinants in the genesis of various epithelial malignancies.

2. Mutational inactivation of the retinoblastoma susceptibility gene has been observed in two of nine human breast cancer cell lines (Lee *et. al.*, 1988). Structural rearrangements of the Rb gene have been reported in both human breast tumor specimens and breast tumor cell lines; these correlate with loss of Rb or expression of aberrant mRNA species (T'ang *et al.*, 1988). In the latter study the low percentage (7% of primary, 25% of cell lines) of alterations indicates that the Rb locus itself may be insufficient for tumor development in mammary tissues. Rb may exert a pleiotropic effect on certain types of cancer, but the action of tissue-specific genetic loci may also be critical.

3. c-*ras* is also commonly overexpressed in human breast cancer as opposed to normal or benign lesions (Lundy *et al.*, 1986). Increases in the tumorigenic capacity of human and mouse mammary epithelial cells have been observed following v-Ha-*ras* or activated c-*ras* transfections (Ochuchi *et al.*, 1986). However activating *ras* oncogene mutations (codons 12, 13, 61) are detectable in only a small percent of primary and metastatic human breast carcinomas. That study concluded such mutations are rarely involved in initiation or metastatic progression, but may reflect terminal stages of malignancy (Rochlitz *et al.*, 1989). A similar study suggested that the initiation of tumor development and any later loss of hormone responsiveness in mammary cancer also represented independent events (Sukumar *et al.*, 1988).

In parent MCF-7 cells, c-*ras* is amplified but not mutated, and a cloned MCF-7 cell line has been stably transfected with v-Ha-*ras* (Kasid *et al.*, 1985). This subline expresses increased levels of laminin receptor (Albini *et al.*, 1986), demonstrates increased turnover of phosphotidylinositol, and secretes higher levels of IGF-1, TGF-α, and TGF-β, but not PDGF. Cells manifest increased tumorigenicity in the absence of estradiol, but retain estrogen responsiveness (ER binding activity, induction of progesterone receptor, slight increase in growth rate). *Ras* transformed cells and non-transformed cells display similar

growth rates under estrogen-deprived conditions; MCF-7 cells transformed with the v-Ha-*ras* oncogene are however less sensitive to further growth stimulation by estradiol, TGF-α, and IGF-1 (Dickson *et al.*, 1987). Both v-H-*ras* transfection and/or estrogen treatment alter the production of secreted and cellular polypeptides by MCF-7 cells; however, only about 20 species are regulated in common by these diverse stimuli (Worland *et al.*, 1989).

4. c-*erb*B-1 (EGFR) is commonly overexpressed in highly aggressive ER- tumors (Sainsbury *et al.*, 1985). Sublines of a human breast cancer cell line with varying degrees of c-*erb*B amplification show a positive correlation between amplification and growth rate; however no correlation between amplification and tumor frequency can be demonstrated in the nude mouse model (Filmus *et al.*, 1987). In addition to the above observations, expression of the EGF receptor is coupled to the progression of cancer from ER+ to ER- status. Several studies suggest an inverse relationship between number of estrogen receptors and EGF receptors in breast tumor tissues (Davidson *et al.*, 1987). However, Zajchowski *et. al.* (1988) notes that mRNA levels for EGFR are comparable in normal and tumor-derived human mammary epithelium (MCF-7 cells).

5. *Int*-1, an oncogene and developmental regulator in Drosophila, is linked to the development of adenocarcinoma in rodent mammary tissue (Rijsewijk *et al.*, 1987a; Rijsewijk *et al.*, 1987b). Tissue specific expression in mammary gland, salivary gland, and male reproductive tissues is observed in transgenic mice when *int*-1 transcription is linked to the MMTV promoter (Tsukamoto *et al.*, 1988). The transcriptional activation of *int*-1 is associated with hyperplasia and major changes in differentiation and development of both mammary tissue and salivary gland. However *int*-1 is more potent as an oncogene in mammary epithelium than in salivary gland, and no correlation between high expression and development of malignancy is seen in the testis.

6. c-*myc* is often rearranged, amplified and/or overexpressed in primary mammary tumors as compared with hyperplastic and normal breast tissue (Escot *et al.*, 1984). Introduction of MMTV-enhancer-*c-myc* constructs into mice zygotes produces mammary cancer in a significant proportion of transgenic female offspring (Stewart, 1984). Although high levels of transgene expression are detected in the entire mammary gland, tumors are monoclonal, suggesting that at least one additional rate-limiting event has occurred. In the

MCF-7 cell line, estradiol increases c-*myc* levels approximately 5-fold within three to six hours of exposure; levels then return to baseline and again increase after twenty-four hours (Santos *et al.*, 1988) Tamoxifen reduces c-*myc* RNA levels by 80% after three to six hours and levels gradually return to baseline after seventy-two hours. Cycloheximide treatment superinduces c-*myc* expression and prevents estrogenic modulation. Evidence in this system also strongly implicates a post-transcriptional mode of regulation.

Amplifications and rearrangements of several oncogenes, including those discussed above, have been reported in human breast tumor specimens. However, a number of clinical investigators advise caution in interpreting the relevance of gross alterations in oncogene structure or expression. (Ali *et al.*, 1988). A recent article suggests that molecular events responsible for loss of hormone dependency in carcinogen-induced rat tumors do not involve gross alterations in several proto-oncogenes (*abl, erb*B-1, *fms, fos, myb, neu, myc*) or in the estrogen receptor itself (Zajchowski *et al.*, 1988). Further, changes in H-*ras* oncogene mRNA expression do not correlate with loss of hormonal dependency (Rochlitz *et al.*, 1989). It shoud be noted that the actual levels of Ras protein expression in these two studies is uncertain. The second report does confirm, as originally reported by Kasid *et al.* (1985) that activated H-*ras* oncogenes partially overcome the requirement for estradiol in MCF-7 growth *in vitro*, but not *in vivo* (at least when expression is driven by the *ras* regulatory elements). In contrast, no consistent differences in mRNA levels of TGF-β, *erb*B-2, or n-*ras* are seen between normal, ER-, or ER+ tumor cells cultured in parallel, although both EGFR mRNA and TGF-α mRNA levels tend to be lower in ER+ cells (Zajchowski *et al.*, 1988). Expression of c-*myc* is slightly lower in tumor cells than in normal cells. An absolute causal relationship between amplification or rearrangement of specific proto-oncogenes has therefore not been established in the case of hormone-dependent breast cancers.

VI. Isolation of Hormonally-Regulated Gene Products

A number of studies of hormone-induced changes in total mRNA complexity have suggested the feasibility of identifying hormonally regulated genes. Testosterone increases the abundance of specific RNA sequences in rat prostate; increases are most evident in the high abundance class of poly(A) RNA (Parker and Mainwaring, 1977). Castration has no effect on the number of different sequences in any class of poly(A) RNA prepared from the seminal vesicle of normal rats and does not alter the concentration of species in the low abundance class (Higgins *et al.*, 1979). Three- and ten-fold decreases in concentration are seen in the abundant and moderate classes respectively. The number of different total poly(A) RNA species in hormone withdrawn chick oviduct is approximately half of that in estrogen-treated chicks or laying hens (Monahan *et al.*, 1976). Five sequences in hormone-treated chicks and hen oviduct are present in excess of 5,000 molecules per cell. These species are apparently absent in hormone-withdrawn chicks. In contrast, heterologous hybridization studies using polysomal poly(A) RNA, indicate that the RNA complexities of mature and hormone-withdrawn oviduct are very similar, and most species of RNA are common to both populations (Hynes *et al.*, 1977). The high abundance messages for ovalbumin, ovomucoid, and lysozyme represent 60% of total mRNA in mature tissue, but only 2% of mRNA in hormone withdrawn oviduct. Estradiol increases the concentration of approximately 85% of RNA species in the high abundance class of poly(A) RNA in rooster liver (King *et al.*, 1979). In that study, no major changes in the complex class were detected. R_0t analyses indicate that both estradiol and tamoxifen induce profound changes in the mRNA population of rat uteri (Aziz *et al.*, 1979). Similarly prolactin administration affects the mRNA population of ZR-75 human mammary cancer cells, increasing both the high and moderate abundance classes (Arya, 1982).

The isolation of hormonally regulated products (either RNA or protein) is a critical issue which limits investigations in many biological systems. Any given RNA sequence can be present in one to 80,000 copies per cell (Bishop *et al.*, 1974), and the current level of detectability in DNA cloning is 0.05% of total mRNA transcripts, or about ten transcripts per cell (Brandis *et al.*, 1986). Most earlier studies on estrogen regulation of gene expression dealt with secreted proteins representing, on induction, 10-70% of cellular

synthetic activities, and more than 1000 mRNA transcripts per cell. In such systems, both mRNA and protein can be readily isolated; consequently preselected RNA species can be used in direct cloning of the relevant gene, or antibodies can be raised to screen expression libraries.

Basal constitutive gene expression also has complicated the isolation of hormonally modulated gene products. Strategies of differential screening require populations differing at least ten-fold in relative levels of a particular RNA transcript. Male *Xenopus* liver vitellogenin and chicken oviduct ovalbumin are not expressed in the non-estrogenized state and are induced to high levels by estradiol (Baker and Shapiro, 1977; McKnight, 1978). However most other gene products known to be modulated by hormones are expressed irrespective of hormone treatment. Moreover, only a small subset of total cell proteins are altered by steroid treatment. For example, glucocorticoid treatment of rat kidney cells affects the expression of about 50 proteins (Ivarie and O'Farrell, 1978). Similarly only a discrete number of proteins can be identified as estrogen-induced in rat uteri (Kamm *et al.*, 1986; Skipper *et al.*, 1980). However, the number of different RNA sequences per cell varies between 7,000 and 40,000; consequently the isolation of hormonally regulated, non-abundant mRNA species has remained technically formidable.

Regardless of the above difficulties, several cDNA libraries have been constructed in hormonally-regulated systems (Berger *et al.*, 1981; King *et al.*, 1984; Lee *et al.*, 1983; Vrontakis *et al.*, 1987). Unfortunately, only one study has directly compared the quality of a cDNA library with its mRNA template (Gordon *et al.*, 1978). Screening of 600 recombinants from a cDNA library constructed with rat uterine mRNA from estrogen-treated immature rats yielded three clones (Travers *et al.*, 1988). The extent (pRU1=10-fold; pRU2 =3-fold; pRU3 =7-fold) and the temporal pattern of induction of these clones differs greatly. All three genes are transcribed constitutively, and in the rat uterus, are stimulated by tamoxifen. Two of the induced species (pRU1 and pRU2) are also detected in MCF-7 cells; levels of both species are increased 2-fold by estradiol, but are essentially unaffected by tamoxifen treatment. Seven clones which appear to be stimulated by estradiol have been isolated from a cDNA library in estrogen-treated GH4C1 cells (Amara *et al.*, 1987). Two clones (1C28 and 1D51) have been examined further; however the extent of induction does not exceed 5-fold.

Estrogen treatment affects a number of specific gene products in MCF-7 cells (table 1). In addition to induction of progesterone receptor (Horwitz and McGuire, 1978), estradiol increases levels of an M_r 24,000 protein (Edwards *et al.*, 1980), M_r 52,000 and 160,000 secreted glycoproteins (Garcia *et al.*, 1985), M_r 32,000 and 37,000 secreted proteins (Sheen and Katzenellenbogen, 1987), and an M_r 46,000 cytokeratin (Brabon *et al.*, 1984). The M_r 37,000 protein is secreted at high levels in the absence of estradiol, and is increased 4-fold by treatment with anti-estrogens. Levels are only slightly decreased by estradiol, but estradiol does antagonize the stimulatory effect of trans-tamoxifen. Levels of the M_r 32,000 protein are increased 10-fold by estrogen treatment. The M_r 52,000 protein has recently been identified as a precursor to a cathepsin D-like protease (Morisset *et al.*, 1986). M_r 52,000 is produced constitutively by ER- mammary cancer cells, is secreted at higher levels by breast cancer cells vs. non-tumorous mammary cells, and has a mitogenic effect on MCF-7 cells (Briozzo *et al.*, 1988; Westley and May, 1987). To date the functions of the other products are unknown.

Levels of different lactic dehydrogenase isozymes (Burke *et al.*, 1978), laminin receptor (Albini *et al.*, 1986), plasminogen activator (Butler *et al.*, 1979), and more recently, a variety of putative growth factors (discussed below) are also altered by estrogen treatment. Estradiol also stimulates a number of specific enzyme activities critical in RNA and DNA synthesis [thymidine kinase (Bronzert *et al.*, 1981); carbamylphosphate synthetase, orotidine decarboxylase, and orotate pyrophosphorylase (Aitken and Lippman, 1985); DNA polymerase (Edwards *et al.*, 1980); and dihydrofolate reductase (Cowan *et al.*, 1982)]. In the cases of thymidine kinase and dihydrofolate reductase, estradiol increases both steady state levels and transcription of the corresponding mRNAs (Kasid *et al.*, 1986; Levine *et al.*, 1985). Estradiol increases cell surface receptor to laminin, cell attachment to laminin-coated artificial membranes, and migration of cells across an artificial membrane toward a diffusible source of laminin (Albini *et al.*, 1986).

Unfortunately, past attempts to construct cDNA libraries from estrogen-treated MCF-7 cells have met with limited success. In general, antibody screening of cDNA libraries has resulted in the isolation of some non-abundant hormonally induced products, including the cDNA for the human DF3 breast carcinoma-associated antigen (Siddiqui *et al.*, 1988), the putative cDNA for an M_r 24,000 protein (Moretti-Rojas and McGuire,

1986), and the cDNA for the M_r 52,000 protein cathepsin D (Westley and May, 1987). DF3 breast carcinoma-associated antigen is not known to be estrogen-regulated; however expression correlates positively with human breast cancer differentiation and ER+ status. The cDNA for DF3 was isolated from a MCF-7 λgt11 library constructed by Chambon and colleagues and has been identified in a number of human mammary cancer cell ine.. This cDNA hybridizes with two mRNA transcripts (4.1 and 7.1 kb) and encodes two glycoproteins (M_r 330,000 and 450,000). The 2.1 kb cathepsin D mRNA is induced 10-15-fold by estradiol. These genes are all expressed constitutively and are only affected quantitatively by exogenous hormone treatment.

Differential screening techniques have also proven partially successful. Three independent laboratories have isolated and verified pS2 as estrogen-regulated using this technique (Masiakowski *et al.*, 1982; Prud'homme *et al.*, 1985; May and Westley, 1986). The function of the pS2 product is unknown, although evidence suggests that neither pS2 nor cathepsin D directly regulate MCF-7 cell growth (Aitken *et al.*, 1985; Davidson *et al.*, 1986; Nunez *et al.*, 1987). The pS2 polypeptide demonstrates considerable sequence homology with a porcine pancreatic secretory product known to inhibit gastrointestinal motility and gastric secretion (Nunez *et al.*, 1987). pS2 expression is directly regulated by estrogen receptor at the transcriptional level, and the 5' sequences which mediate estrogenic induction have been identified (Brown *et al.*, 1984, Roberts *et al.*, 1988). The study of this gene has consequently yielded considerable information concerning the mechanism of estrogen action.

May and Westley (1987, 1988) have identified and characterized eleven cDNAs induced by estradiol in MCF-7 cells, as well as four cDNAs induced in ZR-75 cells; thirteen of these represent distinct species. The temporal regulation and sensitivity to estrogens and anti-estrogens of these clones appear to vary considerably. One of these (pNR-2) has since been identified as pS2, and a second (pNR-100) as the cathepsin D protease. Another (pNR-1) does not correspond to any known sequence in existing data banks. pNR-1 is induced by antiestrogens almost as effectively as by estradiol (50-100-fold). Investigation into the nature of all these genes is being actively pursued.

It can be concluded that both technical and biological constraints apply to the isolation of hormonally altered gene products. In a strategy of differential screening, the

optimum biological system is one in which basal levels of gene transcription are low. From a technical aspect, maximum representation of mRNA transcripts is needed in template RNA, and maximum efficiency in generating a cDNA clone bank is required to isolate low abundance transcripts. Recent advances in cloning and screening strategies have made isolation of such low abundance transcripts feasible. However careful experimental control, and documentation of RNA and cDNA complexity at various stages of library construction is critical in any attempt to clone such transcripts. MCF-7 cells have been utilized in the past to systematically examine the effect of estradiol on specific gene regulation. Undoubtedly isolation of genes regulated by estradiol could yield information of considerable value, generating research tools with which to examine tissue-specific and hormonal regulation; mechanisms of transcriptional, post-transcriptional, and translational control; the role of ER or other estrogen-binding proteins in conferring hormonal responsiveness, etc.

Evidence that discrete gene products are associated with both cell cycle progression and the maintenance of either a quiescent or growing state has been presented previously. The MCF-7 cell line is a biological model which is both clinically and theoretically relevant in approaching the issue of growth regulation. Extensive documentation concerning the action of estradiol as a trophic hormone is available in this system. Regardless of mechanism (direct, or mediated by induction of growth factors or growth factor receptors, estradiol is a critical regulatory element in the control of cellular proliferation in MCF-7 cells. Additionally, recent observations suggest that a simple, controlled experimental manipulation (estrogen deprivation or estrogen treatment) may be sufficient to establish populations from which to isolate such growth-regulatory genes. Isolation of estrogen-induced genes could prove valuable in a number of capacities: 1. therapeutically, as a marker for tumor status or hormonal responsiveness (i.e. DF3 antigen, PR); 2. scientifically, as a tool in investigating the mechanism of tissue specific and hormonal regulation of specific gene products (i.e. PR, pS2); 3. both scientifically and clinically, as possible regulatory molecules influencing cellular growth processes.

INCREASED GROWTH	-	CELL NUMBER
		DNA SYNTHESIS
		RNA SYNTHESIS
		PROTEIN SYNTHESIS
ENZYME ACTIVITY	-	DIHYDROFOLATE REDUCTASE
	-	PLASMINOGEN ACTIVATOR
	-	DNA POLYMERASE
	-	LACTATE DEHYDROGENASE
	-	PURINE AND PYRIMIDINE SYNTHESIS
SPECIFIC PROTEIN	-	PROGESTERONE RECEPTOR
	-	THYMIDINE KINASE
	-	DIHYDROFOLATE REDUCTASE
	-	IGF-1
	-	TGF-α
	-	pS2
	-	52K CATHEPSIN D
	-	24K PROTEIN
	-	46K CYTOKERATIN
	-	28K, 160K GLYCOPROTEINS
	-	TGF-β
	–	LAMININ RECEPTOR
RNA LEVELS / TRANSCRIPTION	-	DIHYDROFOLATE REDUCTASE
	-	THYMIDINE KINASE
	-	24K PROTEIN
	-	pS2
	-	52K CATHEPSIN
	-	PROGESTERONE RECEPTOR
	-	pNR1, pNR2, pNR25
	-	pRU1, pRU2

Table 1. DOCUMENTED EFFECTS OF ESTRADIOL ON MCF-7 CELLS.

SPECIFIC OBJECTIVES

The primary goal of this project was the isolation of cDNAs which are directly or indirectly regulated by estradiol. In essence this investigation can be considered linear; each major undertaking will be dependent on the successful completion of a previous set of defined objectives. The individual elements which comprise this project are therefore depicted in an accompanying flow chart (table 2) and discussed below.

I. Verification of Growth Arrest. An experimental model which stringently distinguishes estrogen-treated (E+) and estrogen-deprived (E-) conditions is critical in any attempt to identify estrogen-regulated gene products. Since we are specifically interested in the trophic effects of estradiol (as defined by increases in cell number, [^{3}H]thymidine incorporation, DNA, RNA and protein content), the well-characterized MCF-7 cell line presents a particularly attractive experimental system. Consequently, the development of conditions under which MCF-7 cell growth is totally dependent on the presence of estradiol becomes a clear priority.

II. Characterization of poly(A) RNA. The second element of this investigation entails confirmation that the poly(A) RNA isolated from estrogen-treated or estrogen-deprived MCF-7 cells demonstrates the appropriate hormonally regulated pattern of gene expression. To this end, known cloned genes (pS2, induced by estradiol; 36B4, unaffected by estradiol; gelsolin, repressed by estradiol) will be employed to probe poly(A) RNA preparations. Additionally the quality of the pooled poly(A) RNA to be used as the template for cDNA synthesis will be assessed in terms of size distribution (Northern blot using radiolabeled cDNA and oligo(dT) probes) and gene representation (R_0t analysis).

III. Construction of the cDNA library. High-quality cDNA libraries are surprisingly rare. The poor success of a number of previous attempts to isolate hormonally-regulated genes in the MCF-7 cell system, as well as in other model

systems, can be attributed not only to a failure to define experimental conditions, but to the difficult technical problems associated with cDNA library construction. The quality of the cDNA library must be evaluated in the same terms as previously described for the poly(A) RNA template. Therefore, Southern blots and C_0t analysis will be employed to verify that the library is representative of the parent RNA population.

IV. Library Screening. The fourth major component of this project is to isolate putative estrogen-regulated clones from a heterogenous pool of λ recombinants. This will necessitate three rounds of differential screening with radiolabeled selected cDNA probes. The single-stranded probes will be synthesized from poly(A) RNA prepared from estrogen-treated or estrogen-deprived cells and hybridized against an excess of poly(A) RNA from estrogen-deprived cells.

V. Characterization of Clones. My end-point is to confirm that, under the experimental conditions established in step I, the expression of any such cloned genes is indeed estrogen-regulated. This will require 1. subcloning from a phage (λgt10) to a plasmid vector (pGEM3Z); 2. determining the pattern of gene expression under several different hormonal regimens (dose response, temporal response, effect of anti-estrogens); 3. identification of any cloned genes by partial sequencing and comparison with gene data banks.

In summation, this project is designed to generate a set of experimental tools which might facilitate further investigations into the molecular mechanisms by which estradiol alters gene expression. Isolation of a gene product which may mediate a trophic response, or is a potential marker for hormonal responsiveness, would be of inestimable clinical value with respect to human breast cancer. The study of such a gene could also give insight into the general mechanisms governing cellular growth. Additionally any cloned hormonally responsive gene, whether involved in cell growth or not, is of interest from the standpoint of understanding the control of gene expression.

I. VERIFICATION OF GROWTH ARREST

II. CHARACTERIZATION OF POLY(A) RNA

- A. NORTHERN BLOT - pS2, 36B4, Mu319 PROBES
- B. NORTHERN BLOT - cDNA, OLIGO(dT) PROBES
- C. R_0t ANALYSIS

III. cDNA LIBRARY

- A. CONSTRUCTION
- B. SOUTHERN BLOT - cDNA, OLIGO(dT) PROBES
- C. C_0t ANALYSIS

IV. LIBRARY SCREENING

V. CHARACTERIZATION OF CLONES

- A. SUBCLONING INTO pGEM3Z
- B. BIOLOGICAL REGULATION
- C. PARTIAL SEQUENCING

Table 2. Organization and Specific Objectives of the Project

MATERIALS AND METHODS

I. Reagents and Solutions (Abbreviations)

A. Reagents

Acrylamide

Actinomycin D (Boehringer-Mannheim, Indianapolis, IN)

Agarose (Seakem GTG agarose; FMC Bioproducts, Rockland, ME)

Agarose (Seakem Nusieve agarose, FMC Bioproducts)

Agarose (LMP agarose; electrophoresis grade, Bethesda Research Laboratories, Gaithersburg, MD)

Adenosine [γ-^{32}P]triphosphate ([γ-^{32}P]ATP; 3000 Ci/mmol; 1 mCi/100 µl; NEN)

S-Adenosylmethionine (SAM; New England Biolabs, Beverly, MA)

AG501-XB(D) (analytical grade mixed bed resin; BioRad, Richmond, CA)

Ammonium persulfate

Ampicillin (Boehringer-Mannheim)

Avian Myeloblastosis Virus Reverse Transcriptase (AMV.RT; 20 U/µl; Seikagaku America Inc., St. Petersburg, FL)

Bacto-agar (DIFCO Laboratories, Detroit, MI)

Bacto-tryptone (DIFCO)

Bis acrylamide

Bovine Serum Albumin (BSA; nuclease-free; 1 mg/ml; BRL)

Bovine serum albumin (BSA; pentax fraction V; Sigma, St. Louis, MO)

Bovine Calf Serum (CS; Gibco, Chagrin Falls, OH)

Bromphenol Blue (Biorad)

Cesium Chloride ($CsCl_2$; optical grade, BRL)

Cobalt Chloride ($CoCl_2$)

Cycloheximide (Boehringer-Mannheim, Indianapolis, IN)

Deoxyadenosine [α-^{32}P]triphosphate ([α-^{32}P]dATP; 400 Ci/mmol; NEN, Boston, MA)

Deoxyadenosine [α-^{35}S]triphosphate ([α-^{35}S]dATP; 400 Ci/mmol; NEN; 10 mCi/ml)

Deoxycytidine [5-^{3}H] triphosphate ([^{3}H]dCTP; 26 Ci/mmol; NEN)

Deoxycytidine [α-^{32}P]triphosphate ([α-^{32}P]dCTP; >3000 Ci/mmol; NEN)

DNA (salmon sperm; Sigma)

DNA size standards (1 kilobase ladder, 123 base pair ladder; BRL)

Deoxyribonucleotides (dATP, dCTP, dTTP, dGTP; Pharmacia P-L Biochemicals, Milwaukee, WI)

Dextran (>200,000 mw, Baker, Philipsburg, NJ)

Dextran sulfate (Sigma)

(4',6-Diamidino)-2-phenylindole dihydrochloride (DAPI; Aldrich, Milwaukee WI)

Dichlorodimethylsilane (Mallinkrodt)

Diethylpyrocarbonate (DEPC; Sigma)

Dimethylsulfoxide (DMSO; Baker, Philipsburg, NJ)

DNase I (Sigma)

Dithiothreitol (DTT; 1M; Promega, Madison, WI)

EcoRI restriction endonuclease (10U/μl, BRL)

EcoRI linkers (Collaborative Research, Lexington, MA)

EcoRI methylase (40 U/μl, New England Biolabs)

EcoRI reaction buffer (10X = 0.1M $MgCl_2$, 1.0M NaCl; 0.1 M Tris·HCl, pH 7.5; BRL)

17β-Estradiol (E_2; Steraloids, Wilton, NH)

Ethanol (EtOH; stored at -20°C)

Fetal calf serum (FCS; Gibco)

Ficoll (Sigma)

Formaldehyde (37% w:v)

Formamide

Gelatin

GeneClean Reagent Kit (Bio 101, La Jolla, CA)

Gigapack Gold Packaging System (Stratagene, San Diego, CA)

Glutamine (100X = 29.2 mg/L, 0.2M; Biofluids, Rockville MD)

Guanidine Isothiocyanate (GuITC; ultrapure enzyme grade; BRL)

Glycerol

Glycogen (20 mg/ml, molecular biology grade, Pharmacia)

Glyoxal (Aldrich Biochemical Co., Inc., Milwaukee, WI)

HEPES (4-[2-Hydroxyethyl]-1-piperazine-ethanesulfonic acid; Boehringer-Mannheim)

HindIII restriction endonuclease (10U/μl, BRL)

Hind III reaction buffer (0.05M NaCl; 0.01M $MgCl_2$; 0.01M Tris·HCl, pH 7.5; BRL)

4-Hydroxy-tamoxifen (OH-TAM, Sigma)

Improved Minimal Essential Medium + 40 mg/L gentamicin + phenol red
(IMEM-I; Gibco, Chagrin Falls, OH or NIH media unit)

Improved Minimal Essential Medium + 40 mg/L gentamicin (IMEM-II; NIH media unit)

2X Improved Minimal Essential Medium (2X IMEM; NIH media unit)

Isoamyl alcohol

Isopropanol

Klenow (1 U/μl, DNA polymerase, large fragment, BRL)

Lysozyme (stored at -20°C)

β-Mercaptoethanol (Et~SH; Sigma)

Nick-Translation Kit (BRL)

Nonidet P40 (BRL)

Norit A (activated charcoal; Sigma)

$Oligo(dC)_{12-18}$ (Pharmacia P-L Biochemicals)

$Oligo(dT)_{12-18}$ (Pharmacia P-L Biochemicals)

Oligo(dT) cellulose (BRL)

Oligolabeling Kit (Pharmacia)

Phenylmethylsulfonyl fluoride (PMSF; Sigma)

Phosphate buffered saline (PBS; Biofluids)

Polyethylene glycol (PEG 6000; Sigma)

Polyuridylic acid [poly(U); stored at -20°C; Pharmacia P-L]

Polyvinylpyrrolidone (PVP; Sigma)

Proteinase K (fungal, Boehringer-Mannheim)

Protoclone λgt10 system (Promega)

Ribonucleotides (ATP, CTP, GTP, UTP; Sigma)

RNA (yeast tRNA; Pharmacia)

RNA standards (BRL, Bethesda, MD; stored at -70°C)

RNase A (Sigma)

RNase A (BRL)

RNasin (placental ribonuclease inhibitor; 30 U/μl; Promega)

S1 Nuclease (166 U/μl; BRL)

Sequenase 2.0 (United States Biochemicals; Cleveland, OH)

Sequenase Kit (United States Biochemicals)

SP6 primer (10 ng/μl; Promega)

Sodium cacodylate (courtesy of Dr. F. Bollum, Dept. of Biochemistry, USUHS)

Sodium citrate ($Na_3C_6H_8O_7 \cdot H_2O$)

Sodium dodecylsulfate (SDS; Sigma)

Sodium lauryl sarkinosate (sarkosyl; Sigma)

Spermidine [spermidine hydrochloride, N-(3-aminopropyl)-1,4-butanediamine; Sigma]

Sulfatase (14.5 U/mg, Sigma)

T4 DNA Ligase (10 U/μl, BRL)

T4 DNA Polymerase (10 U/μl, Boehringer-Mannheim)

T4 Polynucleotide Kinase (7.7 U/μl, Boehringer Mannheim)

T7 primer (10 ng/μl; Promega)

N,N,N',N'-Tetrametylethylenediamine (TEMED)

Terminal Transferase (10 U/μl, BRL)

Tetracycline (Boehringer-Mannheim)

Thymidine, [5-methyl-^{3}H] ([^{3}H]thymidine; 40-60 Ci/mmol; NEN)

Triton X-100

Trypsin A [0.05% trypsin; 0.02% EDTA; phenol red (w:v, Biofluids)]

Trypsin B [0.05% trypsin; 0.02% EDTA (w:v, NIH media unit)]

Uridine [α-^{32}P]triphosphate ([α-^{32}P]UTP; 400 Ci/mmol, NEN)

Xylene cyanol (Biorad)

B. Other Materials

E. coli C600 (courtesy of M. Davis, Stanford U. School of Medicine, Stanford, CA)

E. coli C600 *hfl*A (courtesy of M. Davis, Stanford U. School of Medicine, Stanford, CA)

E.coli HB101 (BRL)

Econo-pac columns (BioRad)

Nytran (0.45μ; Schleicher and Schuell, Keene, NH)

Nitrocellulose filters (BA85 nitrocellulose, Schleicher and Schuell)

Phenylethylimine cellulose thin layer chromatography plates (PEI TLC plates; Baker)

pGEM3Z (Promega)

Select 7L sepharose (603 bp exclusion, 5 Prime - 3 Prime Inc., Paoli, PA)

Select D spin columns (G50 sephadex; 5 Prime - 3 Prime)

Select D spin columns (G25 sephadex; 5 Prime - 3 Prime)

Select 4L sepharose spin columns (5 Prime - 3 Prime)

Select 2L sepharose spin columns (5 Prime - 3 Prime)

Seton easy-seal tube system (PGC, Gaithersburg, MD)

C. Stock Solutions

0.1M Adenosine triphosphate (ATP; prepared as described by Sambrook *et al.*, 1990, p B10; stored at -20°C)

7.5M Ammonium acetate ($NH_4C_2H_3O_2$; NH_4Ac; pH 5.5)

0.5M Ammonium acetate (NH_4Ac; pH 5.5)

DEAE cellulose (Whatman #4057-050 DE52 preswollen microgranular cellulose; Whatman Lab Sales Inc., Hillsboro, OR)

0.01M Deoxynucleotide (dNTP = dATP, dCTP, dTTP, dGTP) stock solutions (prepared as described by Sambrook *et al.*, 1990, p B10; stored at -20°C)

DNase I (1 mg/ml; filtered and stored at -20°C)

2M Disodium phosphate (Na_2HPO_4)

0.1M Ethylene diaminetetraacetate (EDTA), pH 7.0

0.5M Ethylene diaminetetraacetate (EDTA), pH 8.0

Ethidium bromide (EtBr; 10 mg/ml)

2% Gelatin (autoclaved)

1M Magnesium acetate pH 5.5 [$Mg(C_2H_3O_2)_2 \cdot 4H_2O$; MgAc]

1M Magnesium chloride ($MgCl_2$)

1M Magnesium sulfate ($MgSO_4$)

2M Monosodium phosphate (NaH_2PO_4)

20% Potassium acetate ($KC_2H_3O_2$; KAc), pH 5.1

Phenol (phenol: chloroform: isoamyl alcohol; 25:24:1; Sambrook *et al.*, 1990; p B4, B12)

Phenol red (0.2% in TE_2)

Proteinase K (1 mg/ml; filtered and stored at -20°C)

0.01M Ribonucleotide (rNTP = ATP, CTP, GTP, UTP) stock solutions (prepared as described by Sambrook *et al.*, 1990; p B10; stored at -20°C)

RNase A (2 mg/ml; boiled 10 minutes; filtered and stored at -20°C)

SM (0.1M NaCl; 0.01M $MgSO_4$; 0.01% gelatin; 0.01M Tris, pH 7.5; 5.8 g NaCl + 2 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$) + 5 ml 2% gelatin + 10 ml 1M Tris·HCl, pH 7.5 + H_2O to 1 L; autoclaved and stored in 50 ml aliquots)

3M Sodium acetate ($NaC_2H_3O_2$; NaAc), pH 6.0

3M Sodium acetate, pH 5.2

4M Sodium acetate, pH 6.0

4M Sodium chloride (NaCl)

20% Sodium dodecyl sulfate (SDS)

20X SSC [3M sodium chloride; 0.3M sodium citrate ($Na_3C_6H_8O_7 \cdot H_2O$); pH 7.0; Sambrook *et al.*, 1990, p B13]

10X SSC (1.5M sodium chloride; 0.15M sodium citrate; pH 7.0)

STE (0.1M NaCl; 0.001M EDTA; 0.01M Tris·HCl, pH 8.0)

2X STE (0.2M NaCl; 0.002M EDTA; 0.02M Tris·HCl, pH 8.0)

10X TBE (0.89M Tris·HCl pH 8.0; 0.89 M boric acid; 0.02M EDTA)

10% Trichloroacetic Acid (TCA)

2M [Tris(hydroxymethyl)aminomethane] base (Tris base)

2M [Tris(hydroxymethyl)aminomethane] hydrochloride (Tris·HCl)

1 M Tris·HCl, pH 8.0 (Sambrook *et al.*, 1990, p B13)

1 M Tris·HCl, pH 7.5

1M Tris·HCl, pH 7.0

TE_1 (0.01M Tris:HCl pH 7.0; 0.001M EDTA)

TE_2 (0.01M Tris:HCl pH 8.0; 0.001M EDTA)

TE_3 (0.01M Tris:HCl pH 7.5; 0.001M EDTA)

II. Tissue Culture

Solutions

1. CSCS (charcoal-treated, sulfatase-treated bovine calf serum)
2. Medium A = 500 ml IMEM I + 10% FCS + 5 ml 100X glutamine + 10^{-9} M E_2
3. Medium B = 500 ml IMEM II + 2.5% CSCS (12.5 ml) + 5 ml 100X glutamine + 10^{-9}M E_2
4. Medium C = 500 ml IMEM II + 2.5% CSCS (12.5 ml) + 5 ml 100X glutamine + 5 x10^{-7}M E_2
5. Medium E = 100 ml 2X IMEM + 30 ml DMSO + 30 ml FCS + 40 ml H_2O + 2x10^{-9}M E_2; stored at -20° C.
6. 10X DCC (dextran-coated charcoal; 25 g untreated activated charcoal + 2.5 g dextran + 10 ml 1M Tris·HCl pH 8.0 to 1 L final volume; pH 7.0
7. PBS (treated with DEPC and autoclaved)
8. PBS + 0.04% EDTA (treated with DEPC and autoclaved)
9. PBS (treated with DEPC and autoclaved) + 50 µg/ml cycloheximide
10. Estradiol stocks (10^{-2} M - 10^{-9} M in EtOH; stored at -20°C)
11. 4-Hydroxy-tamoxifen stocks (10^{-2}M - 10^{-9}M in EtOH; stored at -20°C)
12. DAPI stock (2 mg/ml in H_2O; stored at 4°C in the dark)

Procedures

1. Preparation of CSCS

100 ml 10X DCC is centrifuged at 12,000g for 10 minutes in 250 ml centrifuge bottles (HB-4 rotor, 8,000 rpm). The charcoal pellet is added to 1 L CS in two 500 ml Ehrlenmeyer flasks and shaken at 56°C for 30 minutes, or at 22°C for 2 hours. The

mixture is recentrifuged at 15,000g for 10 minutes (HB-4 rotor, 9,500 rpm) and transferred to other Ehrlenmeyer flasks. Following treatment with 2 U/ml sulfatase (69 mg/500 ml serum; 37°C; 2 hours), a fresh charcoal pellet is added and the serum is shaken and centrifuged as previously described. The supernatant is filtered through 0.45μ filters, distributed into 100 ml bottles, and stored at -20°C. Prior to addition to medium, the CSCS is refiltered through 0.2μ filters.

2. Cell freezing and thawing

MCF-7 cells are frozen at passages 22-30 and thawed as needed to maintain stock cultures (Lovelock, 1953). For freezing, Medium A is removed from one confluent T75 flask and 5 ml of Trypsin A is added. Afer 1-2 minutes the trypsin is removed and 5 ml of medium E is added. Cells are resuspended by gentle pipetting and 1 ml aliquots are distributed to 1ml cryotubes. The tubes are immediately placed in dry ice and subsequently stored under liquid nitrogen. In order to thaw cells, 10 ml of Medium A is distributed to a T25 flask. One vial of frozen cells is rapidly thawed at 37°C and immediately dispensed into the flask with gentle agitation. After 6 to 8 hours incubation, the original medium is removed and replaced with Medium A.

3. Cell Passage

10-15 ml of medium A or B per T75 is pipetted into empty flasks and flasks are labelled with cell line, passage number, and date. 5 ml of trypsin A or B is added to one confluent T75 flask, removed quickly, and another 2.5 ml.trypsin added and removed. 10 ml medium A or B is then added and pipetted up and down repeatedly to reduce cell clumping. 1 ml of this cell suspension is distributed per new flask to produce a 1:10 dilution.

4. Cell growth

All cultures are grown in a humidified incubator in 5% CO_2 at 37°C. Cultures are routinely tested for mycoplasma infection using a test kit from Flow Laboratories (McLean, VA). Stock cultures are maintained in medium A and passaged every 10 to 14 days with trypsin A. Only passages 24-34 are used for experimental purposes.

Approximately 2 weeks before the start of a given experiment, medium A in 2 subconfluent T75 flasks is replaced with medium B. When confluent these are passaged 1:10 (using trypsin B) into T75 flasks containing 15 ml medium B. 0.75 ml of medium C is added each 3 days to replenish estradiol. These flasks are trypsinized with trypsin B when just subconfluent (~ $5x10^5$ - 10^6 cells /flask), and pooled in 20 ml medium B in a single T75 flask. After verifying that the cells exist primarily as single cell suspensions, cell number is determined at two dilutions. Cells are plated in medium B as follows: multiwell Linbro dishes (28 cm^2 wells) at ~ $2x10^4$ cells/well in 5 ml medium; T75 flasks at ~ $5x10^4$ cells/dish in 15 ml medium; T150 flasks at ~ 10^5 cells/dish in 25 ml medium. In accordance with the experimental protocol described in table 3, medium B is replaced every 2 days. When cells appeared to have doubled 4 times, and 2 days after the last medium change, the medium B in one half of the dishes is replaced with medium C (lacking estradiol), and the other half (E+) of the flasks are harvested. Medium C is subsequently replaced with fresh medium C every 24 hours for the next 3 days. The quiescent cells (E-) are harvested 3 days after the last medium change. Alternative protocols for individual experiments are detailed in the corresponding figure and table legends. Where indicated [^{3}H]thymidine (1 μCi/well in multiwell dishes; 5 μCi/T150 flask) is added to cultures 2 hours prior to cell collection.

5. Cell Harvest and Determination of Cell Number

Medium is aspirated, and PBS:EDTA is added to dishes (1 ml/well in multiwell Linbro dishes; 5 ml/flask in T150 flasks). Cells are gently resuspended by pipetting with a

Pasteur pipette or by scraping with a rubber policeman, pooled by experimental group in 15 or 50 ml conical centrifuge tubes, and collected by low-speed centrifugation (800g, 1 minute). Dishes are washed once with PBS + cycloheximide and once with PBS. The washes are added to the collection tubes and recentrifuged. Samples are resuspended in 10 ml PBS and 9 ml of this material is recentrifuged and used for the preparation of RNA as described in section III.

In order to obtain single cell preparations, the remaining 1.0 ml portion is passed through a 19 gauge needle attached to a 1 ml syringe. Approximately 10^5 - $5x10^5$ cells from the single cell suspension are added to counting vials containing ISOTON III (final volume = 10 ml). Cell number is determined using a model ZBI Coulter counter (Coulter Electronics Inc., Hialeah FL) at the following settings; sample 500 µl; aperture 100µ; lower threshold 11; upper threshold 100+. The remainder of the single cell suspension is centrifuged, resuspended in 500 µl H_2O, and stored at -20°C for determination of protein content, DNA content, and [^{3}H]thymidine incorporation.

6. Measurement of Cell Growth

Samples are thawed on ice and sonicated briefly (setting 3; 5 pulses). DNA content is determined through a modification of the fluorometric technique of Brunk *et al.* (1979). Calf thymus DNA is solubilized in H_2O at 100 µg/ml, sonicated as above, and standard curves are constructed between 0.1 µg and 10 µg. 10 µl DAPI stock solution is diluted to 100 ml with DAPI buffer and 1.5 ml of this reagent mix is added to each standard and unknown tube. Fluorescence values are determined with an FS950 fluorometer (Kratos Inc., Schoeffel Instruments Division, Westwood NJ) at the following settings: sensitivity = 5.0; background = low; lamp = 365 A; emission filter = 450; excitation filter = 350; time constant = 0.6. Protein content is measured with a BioRad assay kit (BioRad, Richmond CA) using the dye-binding method of Bradford (1976). Optical density is recorded with a DU8 spectrophotometer (Beckman Instruments). Both protein and DNA values are calculated using a Hewlett-Packard desktop computer and a specifically developed BASIC program (S. Aitken, unpublished) Acid-precipitable radioactivity is assayed by

precipitation with 10% cold TCA on Whatman GF-A glass fiber filters using a Millipore multisample vacuum apparatus (Lippman *et al.*, 1976; Schrier and Wilson, 1976). Data are normalized per unit DNA, protein, or cell number, and means and standard deviations are again determined using a BASIC program written solely for that purpose (S. Aitken, unpublished).

I. VARIABLES - **CELL PASSAGE (22-35)**

PHENOL RED (-)

ESTRADIOL (10^{-9} M/L)

CELL DENSITY ($2-6 \times 10^5$ cells/dish)

MEDIUM CHANGE (48 hours)

II. PROTOCOL

A. Maintain cultures in Medium B **(E+)**

B. Trypsinize and plate at 10^5 cells per T150 flask in Medium B

C. When cells appear to have doubled 2x, replace the medium in one set of flasks with Medium C **(E-)**. Collect the second set of flasks **(E+)**.

D. Replace medium as above in **E-** flasks at 24 hour intervals for the next 3 days. Collect E- flasks 48 hours after the last medium change.

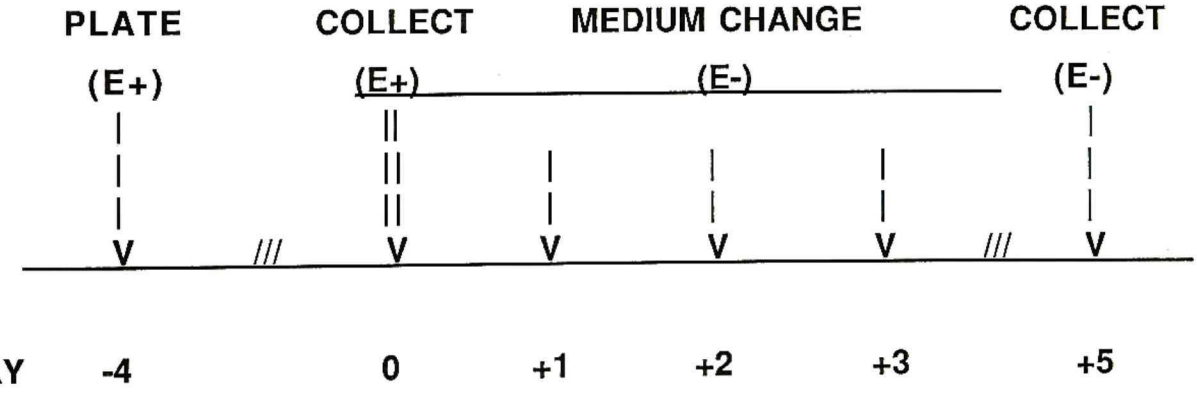


Table 3. Growth of Estrogen-Treated and Estrogen-Deprived MCF-7 Cells.

III. Isolation and Analysis of RNA (All solutions used for nucleic acids are either treated with DEPC or prepared in DEPC-treated, autoclaved H_2O unless otherwise specified)

A. Total RNA for Cytoplasmic Dot and Slot Blots (White and Bancroft, 1982; Papavasliou *et al.*, 1986)

Solutions

1. 0.1M Tris·HCl, pH 7.0
2. TENP (2.0 ml Tris·HCl, pH 7.5 + 0.4 ml 0.5M EDTA + 1 ml NP40 + 16.6 ml H_2O; filtered)
3. 2X storage buffer (10 ml 4M NaCl + 40 ml 0.1M Tris·HCl, pH 7.0 + 20 ml 20% SDS + 130 ml H_2O; filtered)
4. 5% Nonidet P40 (filtered)
5. 0.2M NaCl:EtOH (0.5 ml 4M NaCl + 9.5 ml H_2O + 20 ml ETOH)
6. 0.001M EDTA, pH 7.0

Procedure

Medium is aspirated and 1 ml PBS + 0.04% EDTA is delivered to each well. Cells are collected from the surface of the plate with a Pasteur pipet by squirting the surface of the plate, the suspension is transferred to a 1.5 ml microfuge tube, and centrifuged (12,000g, 5 seconds). The plates are rinsed successively with 1 ml PBS + 50 µg/ml cycloheximide and 1 ml PBS. The cells are resuspended in 50 µl TENP and mixed on ice for 5 minutes. Following addition of another 5 µl NP40, the incubation is continued for 5 minutes and the nuclei are pelleted (12,000g, 2.5 minutes). The pellets are saved for determination of DNA content and [^{3}H]thymidine incorporation. 50 µl of supernatant (cytoplasmic extract) is transferred to a microfuge tube. In the case of multiwell dishes (usually 4 well), extracts are pooled in a single tube of 200 µl final volume. An equal

volume of 2X storage buffer is added (i.e. 200 µl) and extracted 2 times with 200 µl phenol; the phenol phases are then reextracted with 100 µl TE_1 + 100 µl 2X storage buffer, and the aqueous phases are pooled. The phenol extraction and back-extraction are repeated. 20 µl 4M NaCl, 50 µl 0.5M NH_4Ac, and 2.5 volumes of EtOH are then added, and the tubes are stored at -20°C for a minimum of 48 hours. These are centrifuged 15 minutes, washed twice with 50 µl NaCl:EtOH and twice with 50 µl EtOH, and dried under vacuum. The RNA pellets are stored at -70°C in 50 µl 0.001M EDTA.

B. Large Scale RNA Purification (Chirgwin *et al.*, 1979; Glison *et al.*, 1974)

Solutions

1. 4M guanidine isothiocyanate (GuITC)
2. 0.1M sodium citrate, pH 7.0 (NaCi)
3. 10% sarkosyl (stored at 0-4°C)
4. CHAOS buffer (0.005M 0.1M NaCi; 0.1M mercaptoethanol; 0.5% sarkosyl; 1.0M GuITC; stored at 0-4°C)
5. 5.7M $CsCl_2$ (9.58 g $CsCl_2$ + 2 ml 0.1M EDTA + 8 ml H_2O; filtered)
6. 0.01M Tris pH 7.0; 0.005M EDTA; 0.2% SDS
7. Chloroform:1-butanol (4:1; v:v)

Procedure

The medium is aspirated and cells are collected in PBS + EDTA by centrifugation at 1000g for 2 minutes in a clinical centrifuge. The flasks and cell pellets are rinsed successively with PBS + cycloheximide and with PBS. Cell pellets are resuspended in 10 ml PBS and 300 µl is removed for determination of protein, RNA and DNA content. The cells are repelleted and resuspended in 5 volumes (5 ml/1 ml pellet or 5 ml/g of tissue) of CHAOS buffer. This is vortexed thoroughly, and 1 g solid $CsCl_2$/2.5 ml solution is added

when the cellular material appears thoroughly dispersed. This again is mixed until no crystals are visible.

A solution of 6 g $CsCl_2$/15 ml CHAOS buffer is prepared and a volume which will amount of about 1/3 of the final tube volume (i.e. 3.3 ml for a 10.5 ml tube) is added to each centrifuge tube. No drops should remain on the sides of the tube. The tube is then filled with the cell suspension (in swinging buckets to within 1/8 inch of the top; in Seton tubes to the lower rim), avoiding bubbles. Tubes are balanced within 0.1 g, capped, mounted in the rotor, and centrifuged at 80,000g for 16-20 hours at 20°C (Beckman L5-50 ultracentrifuge; 35,000 rpm in 75Ti rotor; 40,000 rpm in 50Ti; 35,000 rpm in SW50.1; 25,000 rpm in SW27). The supernatant is removed with a transfer pipette. The pellet should now be visible as a clear and shiny gelatinous material. The walls of the tube are wiped clean, and the pellet is dissoved in 2 ml Tris:EDTA:SDS. This is transferred to a 50 ml conical and extracted with an equal volume of chloroform-butanol, shaking ~10 minutes on an inverter. The upper (aqueous) phase is removed to a 30 ml RNase-free corex tube (treated with 10% dichlorodimethylsilane) and the extraction is repeated 2 times, pooling aqueous phases.

0.1 volumes (0.6 ml) of 3M NaAc pH 5.2 are added and mixed with 2.2 volumes (14.6 ml) ETOH. This is stored at -20°C for 2 hours and then centrifuged at 12,000g for 30 minutes at 4°C (Sorvall RC-5B centrifuge, SA600 rotor, 9,000 rpm). The pellet is dissolved in 1.0 ml H_20 or 0.001M EDTA and reprecipitated as above. The RNA pellet is then resuspended in 100 µl 0.001M EDTA. 1 µl RNA is diluted to 100 µl with H_20, and the spectrophotometric readings at 280 and 260 are recorded. The R value and the total amount of RNA are calculated [1 mg RNA/ml = 20 (OD_{260}) ; R (OD_{260}/OD_{280}) = ~2.0]. The RNA is divided into small portions as desired for gels and poly(A)RNA selection and stored with 3M NaAc and EtOH at -20°C as described above.

C. Poly(A) RNA Selection (Aviv and Leder, 1972)

Solutions

1. 1N NaOH
2. 0.1N NaOH (filtered)
3. 5M lithium chloride (LiCl)
4. Binding Buffer (0.5M LiCl, 0.01M NaAc pH 6.0, 0.001M EDTA, 0.1% SDS; 10 ml LiCl + 250 µl 4M NaAc pH 6.0 + 200 µl 0.5M EDTA + 500 µl 20% SDS + 89 ml H_20; filtered)
5. Oligo(dT) cellulose (0.5 g/5.0 ml binding buffer, stored at 0-4°C)
6. 0.001M EDTA, pH 7.0

Procedure

The econo-pac column is dipped into 1N NaOH and rinsed 10 times with distilled H_20. 2 ml of the oligo(dT) cellulose suspension is added to the column and allowed to drain. This is followed by 10 ml 0.1N NaOH, 10 ml H_2O, and 10 ml of binding buffer. The pH of the eluted buffer should not be above 7.0.

The RNA (stored with NaAc and EtOH at -20°C) is collected by centrifugation in a microfuge (12,000g, 20 minutes) at 4°C, resuspended in 2.0 ml H_20, and the previous OD readings are confirmed. The tube is heated in boiling H_20 for 2 minutes and chilled quickly in ice. The RNA is adjusted to binding salt conditions with 200 µl LiCl, 5 µl 4M NaAc pH 6.0, 4 µl EDTA, 10 µl SDS, and 780 µl H_20 per ml of RNA, and applied to the column at 0-4°C with a sterile transfer pipet. The eluate is collected in a 15 ml conical tube and is immediately reapplied to the column. This eluate is collected and labeled as poly(A-). The column is then washed with 10 ml of binding buffer which is collected into a 50 ml conical tube. After transferring the column to room temperature, the poly(A+) RNA is eluted with 3 x 2.0 ml 0.001 M EDTA into a 15 ml conical tube. The column is washed successively

with 10 ml 0.1 N NaOH, 10 ml H_20 and10 ml binding buffer to eliminate any residual rRNA. The pH is again tested after these washes. The poly(A+) eluate is readjusted to binding buffer conditions and reapplied to the column at 0-4°C. This eluate is pooled with the previous poly(A-) fraction. The column is then rinsed with 10 ml binding buffer, transferred to room temperature, and the poly(A+) fraction is eluted as described above into a siliconized (10 % dichloromethylsilane) 30 ml tube.

Both the poly(A+) and poly(A-) eluates are precipitated with 3M NaAc pH 5.2 (50 µl /ml RNA) and 2.5 volumes of cold EtOH at -20°C overnight (note 30 µg/ml RNA is necessary for efficient precipitation; glycogen can be used as a carrier). The RNA is collected by centrifugation as described previously, and the pellets are dissoved in 100 µl 0.001 M EDTA. 1 µl is diluted to to 400 µl and the R value and total RNA content are determined as described above. NaAc and EtOH are added to the RNA as described above and the poly(A) RNA is stored at -20°C as described above in appropriate quantities for Northern analyses, hybridization studies, and cDNA synthesis. The column is washed with 0.1 N NaOH, H_20, and binding buffer. The oligo(dT) cellulose is poured into a conical tube and stored in binding buffer at 4°C. This can be reused.

G. Glyoxal Denaturing Gels and Northern Blotting (Favaloro *et al.*, 1980; Reed and Mann, 1985;, McMasters and Carmichael, 1974)

Solutions

1. 4X Loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol, 0.01M phosphate buffer pH 6.8, 0.002 M EDTA, 10% glycerol; autoclaved)
2. glyoxal (2 ml of glyoxal + 1 g of AG501-XB(D) resin is vortexed for 5 minutes and centrifuged; the supernatant is treated with resin twice more and is then filtered thru a 10 ml syringe with a glass fiber filter to deionize the glyoxal; this is stored at -70° in 100 µl aliquots)
3. 1.0M phosphate buffer, pH 6.8 (100 ml 2M Na_2HPO_4 + 100 ml 2M NaH_2PO_4 to 400

ml final volume; autoclaved)

4. 0.01M phosphate buffer, pH 6.8 (5 ml 2M Na_2HPO_4 + 5 ml 2M NaH_2PO_4 to 1 L final volume; autoclaved)
5. glyoxylating solution (125 µl DMSO, 42 µl glyoxal, 3 µl 1M phosphate buffer)

Procedure

1-3 µg RNA (stored with NaAc and EtOH at -20°C as described above) is collected by centrifugation in a microfuge at 4°C (12,000g, 20 minutes) and resuspended in 3 µl H_2O + 7 µl glyoxylating solution; this is then heated at 50°C for 1 hour. 4 µl loading buffer is added just before electrophoresis. A 1% agarose gel is prepared by mixing 1.0 g agarose, 1 ml 1M phosphate buffer, and 100 ml of H_20. This is heated, cooled, and poured into a BioRad DNA subcell horizontal gel. 0.01 M phosphate buffer is added to the chambers after 1 hour, the samples are loaded, and the gel is run for 15 minutes at 100V without buffer circulation. The circulation is then begun and the gel is run about 2 hours, or until the bromphenol blue dye has migrated 9 cm. Molecular weight marker lanes are cut out, stained in the dark for 15 minutes (50 µl EtBr in 100 ml 0.5 M NH_4Ac), and destained in the dark 15 minutes (0.5 M NH_4Ac, pH 5.1). The gel is photographed (type 57 film, red + yellow filters) on a shortwave UV transilluminator using a ruler to determine actual migration distance.

H. Northern Blots and 'Slot Blots'

To prepare a Northern blot, a Nytran membrane (cut slightly larger than the gel) is first saturated with H_20. 3 pieces of 3MM paper saturated with 10X SSC, the gel (no air bubbles should be visible below the gel), the Nytran membrane (again no air bubbles), 3 pieces of 3MM filter paper saturated with 10X SSC, a 1 inch stack of paper towels, and a light weight to compress all layers are successively placed on a smooth flat surface (generally a pyrex dish). The gel is surrounded with saran wrap or parafilm to prevent the paper towels from coming in contact with the lower stack of millipore paper, and

approximately 150 ml of 10X SSC is added to the dish. Following transfer (12 hours minimum) the Nytran is briefly rinsed in 5X SSC to remove bits of gel or particles from the membrane, air-dried for 30 minutes, and exposed to UV light for 90 seconds. These membranes can be stored dessicated at 4°C for months.

Slot blots are performed essentially as described by White and Bancroft (1982). The Minifold II slot blotting apparatus and support paper are provided by Schleicher and Schuell. Nytran membrane is cut to fit the blotting apparatus and is prewet with 0.025M phosphate buffer, pH 7.0. RNA (1-5 μg total RNA; 0.1-1 μg poly(A) RNA) samples are serially diluted with phosphate in a 96 well microtiter plate to yield 3 concentrations in a final volume of 150 μl. Samples are applied to the slots under minimal suction and washed through with 200 μl of 0.025M phosphate buffer. The blots are air dried for 30 minutes and exposed to UV light as described above.

IV. Isolation and analysis of DNA

A. Large Scale Preparation of Plasmid DNA (Garger et al., 1983; Birnboim and Doly, 1985)

Solutions

1. Super Broth (3 g bactotryptone + 6 g yeast extract + 1.25 ml glycerol + 225 ml H_2O; autoclaved)
2. 1 M potassium phosphate, pH 7.6 with phosphoric acid (K_2HPO_4; autoclaved)
3. 0.1M potassium phosphate, pH 7.6
4. Ampicillin (200X stock; 8 mg/ml; filtered and stored at -20°C)
5. Tetracycline (200X stock, 2.5 mg/ml; filtered and stored at -20°C,)
6. Lysis Buffer (0.025M Tris, pH 8.0; 0.05M EDTA; 1% glucose)
7. 5.0 M potassium acetate, pH 4.8 [3M potassium acetate (KAc) + 2M acetic acid (HAc)]
8. NaOH/SDS (0.2N NaOH + 1% SDS; 2 ml 10N NaOH + 5 ml 20% SDS + 93 ml H_2O, made weekly)
9. 1.470 g/ml $CsCl_2$ (optical grade in TE_2, η = 1.378; ρ = 1.47)

Procedure

25 ml of 0.1M potassium phosphate and the appropriate antibiotic are added to 225 ml of super broth, and 10 ml of this medium is innoculated with a single bacterial colony. The culture is shaken at 37°C until in stationary phase. Then the entire 10 ml culture is added to 240 ml of super broth with the appropriate antibiotic and shaken overnight at 37°C (speed setting 6). Cells are collected by centrifugation in a 250 ml polypropylene bottle at 1500g (GS-3 or HB-4 Sorvall rotors, 3000 rpm, 30 minutes, 4°C). The supernatant is discarded, and the walls of the bottle are carefully wiped. The bacteria are

then suspended in 10 ml of lysis buffer on ice. 20 ml of NaOH/SDS is added and mixed gently. After 10 minutes, 15 ml cold KAc is added, mixed gently and incubated for 5 minutes. This lysate is centrifuged at 12,000g for 30 minutes at 4°C (Sorvall HB-4 rotor, 8000 rpm; SA600 rotor, 9000 rpm). The supernatant is filtered through 2 layers of cheese cloth into a second centrifuge bottle. An equal volume of isopropanol is added, samples are placed in the freezer (-70°C for 30 minutes; -20°C overnight), and the precipitate is collected by centrifugation at 12,000g (HB-4 rotor, 7000 rpm, 30 minutes, 4°C). The pellet is resuspended in 10 ml TE_2 and transferred to a 30 ml corex tube. 5 ml 7.5M NH_4Ac is added, samples are incubated on ice for 20 minutes, and tubes are centrifuged at 12,000g (SA600 rotor, 9000 rpm, 30 minutes, 4°C). The pellet is discarded, and the DNA in the supernatant is then precipitated with 30 ml of cold EtOH. The DNA pellet is recovered by centrifugation as described above and is dissolved in 2.4 ml of TE_2. 4.2 g of $CsCl_2$ is added to the DNA and dissolved. 6 ml of $CsCl_2$ solution is placed in the bottom of a 10.5 ml ultracentrifuge tube. 0.4 ml of EtBr is then added to the DNA and this is layered carefully under the 6 ml of $CsCl_2$ solution. The tubes are filled with $CsCl_2$ solution (1.470 g/ml), capped, balanced and centrifuged at 250,000g for 5 hours at 20°C (Beckman 75 Ti rotor, 60,000 rpm). The plasmid band (1-2 ml) is recovered by puncturing the tube with a 20 gauge needle attached to a 5 ml syringe just below the band and withdrawing the plasmid into a 15 ml conical polypropylene tube.

The EtBr is removed by repeated extractions with isoamyl alcohol. The DNA is diluted with 3 volumes of TE_2, transferred to a 30 ml corex tube, and precipitated with 1/80 volumes of 4M NaAc pH 6.0 and 2 volumes of EtOH (30 minutes at -70°C; overnight at -20°C). The plasmid DNA is collected by centrifugation at 12,000g as described previously. The DNA pellet is dissolved in 0.4 ml TE_2 in a 2 ml screw top microfuge tube and reprecipitated with 0.2 ml 7.5M NH_4Ac and 1.2 ml EtOH. After centrifugation at 12,000g the DNA is dried under vacuum and resuspended in 0.5 ml TE_2.

C. Plasmid Minilysate (Holmes and Quigley, 1981)

Solutions

1. RNase A (2.0 mg/ml)
2. STET (8 % sucrose, 5 % Triton X, 0.05M EDTA, 0.05M Tris pH 8.0; 8 g sucrose + 5 ml Triton X-100 + 10 ml 0.5M EDTA + 5 ml 1M Tris·HCl pH 8.0 + H_2O to 100 ml)
3. 10 mg/ml lysozyme in STET (dissolved just before use)
4. LB (10 g bacto-tryptone + 5 g bacto-yeast extract + 7.5 g NaCl + H_2O to 1 L; autoclaved)

Procedure

A 5 ml culture of the desired bacteria is grown overnight in 50 ml conical tubes containing 5 ml LB + antibiotic. Cells are centrifuged at 3000g (3000 rpm) in a clinical centrifuge for 10 minutes and the pellet is resuspended in 500 µl STET in a 1.5 ml microfuge tube. 50 µl of lysozyme is added and the tube is heated to 100°C for 60 seconds in a temperature block. The sample is immediately centrifuged (12,000g, 10 minutes, 22°C). The gelatinous pellet is removed with a sterile toothpick and discarded. The DNA is precipitated with 400 µl isopropanol (5 minutes in a dry ice bath). After thawing, the tube is centrifuged for 5 minutes and the supernatant is discarded. The pellet is dissolved in 300 µl TE_2 in a 65°C water bath, 150 µl 7.5M NH_4Ac is added, and tubes are placed on ice for 20 minutes to precipitate *E. coli* DNA and high molecular weight RNA. After centrifuging for 10 minutes at 12,000g, the supernatant is removed to a new tube, 1 µl of RNase A is added, and samples are incubated for 15 minutes at 37°C. The samples are extracted with an equal volume of phenol and the aqueous phase is tranferred to a 2 ml screwtop tube. This is filled with EtOH and placed in the -70°C freezer for 30 minutes.

Following centrifugation, the pellet is dissolved in 50-100 µl of TE_2. The $OD_{260/280}$ is recorded and 1 µl of the plasmid is examined by electrophoresis on a 1% agarose gel.

D. Large Scale λ Purification (Yamamoto *et al.*, 1970; Maniatis *et al.*, 1982, p.8

Solutions

1. 20% maltose (filtered through 20µ filters)
2. $CsCl_2$ (η = 1.3768; ρ =1.45; ~ 30g/42.5 ml SM)
3. $CsCl_2$ (η = 1.3815; ρ = 1.50; ~ 33.5 g/41 ml SM)
4. $CsCl_2$ (η = 1.3990; ρ = 1.70; ~ 47.5 g/37.5 ml)

   Note: the refractive indices are verified using a refractometer and solutions are diluted with SM as necessary.
5. 1.5 g/ml $CsCl_2$ in SM

Procedure

The host strain bacteria are innoculated into a 250 ml flask containing 100 ml LB and 0.2% maltose. Cells are grown overnight, centrifuged, and resuspended in 10 ml SM. Cells are infected with ~10^7 bacteriophage and incubated at 37°C for 20 minutes with intermittent shaking. 2.5 ml of each culture is dispensed into 250 ml of LB medium + 0.1M $MgSO_4$ in 1 liter flasks and incubated at 37°C with vigorous shaking. The OD_{600} is monitored at 30 minute intervals. When the OD abruptly begins to drop (1.0 to 0.2), 5 ml $HCCl_3$ is added to each flask and shaking is continued. Lysates can be stored overnight at 0-4°C. The chilled lysates are brought to 22°C and DNase I and RNase A are added to a final concentration of 1 µg/ml. Following a 30 minute incubation, 14.8 g NaCl is added, dissolved by swirling, and the flasks are kept on ice for 1 hour. Solid PEG is added to 10% and dissolved by slow stirring on a magnetic stirrer at room temperature. This is then

cooled for 1 hour on ice and transferred to 250 ml polypropylene centrifuge bottles. After a ten minute centrifugation at 12,000g (HB-4 rotor, 7000 rpm, 4°C), the supernatant is discarded. The phage pellet is gently resuspended in SM (4 ml/250 ml culture), and 0.5 g of solid $CsCl_2$ is added per ml of suspension.

$CsCl_2$ solutions of decreasing density are layered on top of each other in ultraclear centrifuge tubes andthe interface between the ρ = 1.50 layer and the ρ = 1.45 layer is marked on the outside of the tube. Samples are centrifuged at 60,000g for 2 hours at 4°C (SW27 rotor, 22,000 rpm). The phage band is collected into a 15 ml conical tube using a plastric pasteur pipette from above. 1.5 g $CsCl_2$/ml is added to the suspension, dissolved, and the phage are transferred to a second ultraclear tube. These tubes are filled with 1.5 g/ml $CsCl_2$ solution and are centrifuged at 200,000g for 24 hours at 4°C (SW27 rotor, 38,000 rpm). The band is collected as described above and the phage are dialyzed twice against a 1000-fold volume of TE_2 for 1 hour at room temperature. The suspension is then transferred to a 15 ml conical centrifuge tube and treated with 50 μg/ml proteinase K + 0.5% SDS for 1 hour at 37°C. The samples are then extracted successively with phenol; phenol:$HCCl_3$ (1:1), and $HCCl_3$. The DNA is precipitated with 1/2 volume of 7.5M NH_4Ac and 2 volumes EtOH overnight at -20°C.

E. Small Scale λ Purification (Helms *et al.*, 1985)

Solutions

1. 1M magnesium acetate (MgAc; filtered)
2. 3M potassium acetate pH 5.1 (KAc; filtered)
3. lysate diluent (0.01M Tris.HCl, pH 8.0; filtered)
4. chase buffer (0.01M Tris.HCl, pH 8.0; 0.01M MgAc; 0.06M NaAc; filtered)
5. elution buffer (0.01M Tris.HCl, pH 8.0; 0.05M MgAc; filtered)
6. phage diluent buffer (0.01M Tris.HCl, pH 8.0; 0.02M $MgCl_2$; filtered)

Procedure

A 5 ml lysate is transferred to a 15 ml corex tube. RNase A (5 µl) and DNase I (5 µl) are added and the samples are incubated for 30 minutes at 37°C. 292 mg NaCl is added and the samples are then incubated for 1 hour on ice. After centrifugation for 10 minutes at 4°C (SA600 rotor, 8500 rpm = 11,000g), the supernatant is transferred to a second 15 ml corex tube. 500 mg PEG (10% final concentration) is added, dissolved by swirling and incubated on ice for 1-2 hours. The pellet is collected by centrifugation as described above, resuspended in 0.5 ml H_2O and extracted 3 times with 0.5 ml $HCCl_3$. The volume is adjusted to 7 ml with lysate diluent.

Approximately 20 g of DE52 cellulose is washed with 0.1N HCl in a 50 ml conical until the pH of is < 2 (2 batch washes). The cellulose is then batch-washed 5-6 times with lysate diluent (pH increases ~ 1 Unit with each wash), and stored at 0-4°C. in a 1:1 slurry with lysate diluent. 4 ml of this slurry is pipetted into an econo-pac column. The packed bed volume should be ~ 2 ml and the flow rate ~ 0.2 ml/ minute. 7 ml of lysate is poured over the column and the eluate is discarded. 5 ml of chase buffer and 0.75 ml of elution buffer are then applied and these eluates are again discarded. 1 ml of elution buffer is finally added and this is collected in a 1.5 ml microfuge tube.

The samples are then treated with 4 µl proteinase K and 40 µl SDS and incubated at room temperature for 5 minutes. On addition of 167 µl KOAc a precipitate will form. The precipitate will dissolve when heated to 88°C for 20 minutes and reform on cooling to 0°. After centrifugation (12,000g, 15 minutes, 4°C), the 1.2 ml supernatant is divided and dispensed into 3 microfuge tubes. The λ DNA is stored at -20°C for 1 hour with 2 µl glycogen as carrier and 0.8 ml isopropanol. The sample is centrifuged as above, and the pellet is washed with 80% EtOH and dried under vacuum. DNA is suspended in 50 µl TE_2, and 1 µl samples are taken for $OD_{260/280}$ readings and gel electrophoresis.

F. Southern and 'Dot' Blots (Southern, 1975; Southern, 1979)

In the case of Southern blots, molecular weight standards (1 kilobase ladder; 123 base pair ladder) lanes are cut out following electrophoresis, stained with EtBr, and photographed as previously described for RNA glyoxal gels. The remainder of the gel is then soaked at room temperature with gentle shaking in:

a. 0.25N HCl , 10 minutes

b. 1.5M NaCl/ 0.5N NaOH, 30 minutes

c. 1.5M NaCl/ 0.5M Tris·HCl, pH 7.5, 30 minutes

The gel is blotted by capillary action overnight, layering in order: 10X SSC, wicks of 3MM paper, support, gel , Nytran membrane (cut to fit the gel and prewet in 10X SSC), 3 pieces of 3MM paper, a 2 inch stack of paper towels, and a light weight. The blot is briefly rinsed in 5X SSC to remove any agarose, air dried and DNA is cross-linked to the membrane (30 seconds under UV light).

For dot blots, a Nytran membrane is cut to fit the Schleicher and Schuell Minifold dot blotting filter apparatus. 100 µl of 0.1M NH_4Ac (50 µl of 7.5M NH_4Ac diluted to 37.5 ml) is applied to each well under minimum vacuum. DNA samples (1-5 µl) are diluted into 100 µl of 0.1N NaOH in 96 well microtiter plates. The NaOH is neutralized with 100 µl of 0.2M NH_4Ac (100 µl 7.5M NH_4Ac diluted to 37.5 ml), and the sample is immediately applied to the filter. The DNA will renature within seconds. The samples are washed through the filter under minimum suction with 100 µl of 0.1M NH_4Ac. Filters are air-dried and DNA is cross-linked to the Nytran as described above.

V. cDNA Library Construction (figure 1)

A. First Strand cDNA synthesis (Retzel *et al.*, 1980; Houts *et al.*, 1979; Berger *et al.*, 1983)

Solutions

1. 0.1M dithiothreitol (DTT; stored at -20°C)
2. 2 ng/ml Actinomycin D, filtered and stored at -20°C)
3. 0.25M magnesium chloride ($MgCl_2$)
4. Oligo(dT) (1 µg/ml, stored at -20°C)
5. 1M Tris·HCl, pH 8.3 at 43°C
6. 1 M KCl
7. 0.1M EDTA, pH 8.0

Procedure

Poly(A) RNA is centrifuged and resuspended in H_2O at a volume of 1 µg/µl. 5 µg is used as the template for library construction. 2 µl of [^{3}H]dCTP is dried under nitrogen and resuspended in 5 µl H_20. 1 µl is counted and the amount of label is then adjusted to ~500,000 cpm/µl.

5 µl RNA, 4 µl 1M Tris·HCl pH 8.3, 4 µl 1M KCl, 1 µl 0.25M $MgCl_2$, 2 µl oligo(dT), 10 µl dNTP mix (combine 3 µl each of 0.01M dATP, dCTP, dGTP, dTTP stocks), 2 µl RNasin, 1 µl Actinomycin D, 1 µl [^{3}H]dCTP, and 4 µl AMV·RT are then added in order to a 1.5 ml microfuge tube. The reaction volume is adjusted to 43 µl with H_2O and this is incubated at 43°C for 1 hour. An additional 2 µl enzyme is then added and the incubation is continued for a second hour. The reaction is terminated with 5 µl 0.1M EDTA. 1 µl is removed and diluted to 5 µl; 1 µl of this is used to assay the

radioactivity incorporated into DNA by TCA precipitation on Millipore GF/C filters, and 1 µl is employed for ascending thin-layer chromatography on PEI cellulose strips with 0.01N HCl. The recovery of single-stranded cDNA is calculated as described below.

The specific activity of [^{3}H]dCTP = 500,000 dpm/(10^{-2} M/L dCTP x $2.5x10^{-6}$L) = 500,000 dpm/$2.5x10^{-8}$ moles = 20,000 dpm/nmole dCTP incorporated into DNA . 4 nucleotides are actually incorporated into DNA. Therefore the specific activity of single-stranded DNA = 5,000 dpm/nmole. Since the average molecular weight of 1 nmole of nucleotide = 324 ng, the specific activity of single-stranded cDNA = 15.43 dpm/ng.

Unincorporated nucleotides are then removedwith Select 7L sepharose columns following manufacturer instruction protocols (5 Prime -- 3 Prime, Inc.). The cDNA is precipitated with 0.1 volumes of 4M NaAc and 2.5 volumes of EtOH (1 µl glycogen as carrier) and air- dried. The pellet is resuspended in 54 µl TE_2 and any remaining poly(A) RNA is hydrolyzed by incubating at room temperature with 0.1N NaOH (6 µl 1N NaOH) for 15 minutes. The pH is readjusted to 5.5 with 6 µl 1M NH_4Ac, and the volume is increased to 100 µl with TE_2. The sample is deproteinized by 2 successive extractions with phenol and ether, and precipitated in dry ice:EtOH with 11 µl 4M NaAC, and 250 µl EtOH (1 µl glycogen as carrier). The pellet is carefully washed with 100 µl of 80% EtOH, reprecipitated, and dried under vacuum. The sample is adjusted to 11 µl with H_20; 1 µl is transferred to a fresh tube and diluted to 10 µl with H_20 ; 1 µl of this is then counted to determine final first strand recovery, and 9 µl is returned to the first tube.

Terminal transferase is subsequently employed to add deoxyguanidine residues to the 3' end of the single-stranded cDNA. 19 µl DNA (see above), 2 µl 1M sodium cacodylate, 3 µl 0.01M dGTP, 1 µl 0.25M $CoCl_2$, and 1 µl terminal transferase are incubated at 37°C for 2 hrs. The reaction is terminated with 1 µl 0.1M EDTA + 23 µl TE_2, and the sample is heated at 70°C for 2-5 minutes and quickly chilled on ice.

B. Second Strand cDNA Synthesis

Solutions

1. Oligo(dC) (0.6 μg/ml, stored at -20°C.)
2. 10X T4 DNA polymerase buffer (0.5M Tris·HCl, pH 8.0; 0.1M $MgCl_2$; 0.25M KCl)

Procedure

1 μl [α-^{32}P]dCTP (1μCi) is diluted to 20 μl with H_2O and 1 μl of this is counted. The amount of radioactivity is then adjusted to 10^6 dpm/μl and 10^6 dpm are added to a 0.5 ml microfuge tube labeled as tube B. The tube containing the first strand cDNA from the previous sequence of reactions is correspondingly labeled as tube A. 1 μl oligo(dC) is then added to tube A and this is heated at 100°C for 1 minute, transferred to a 37°C H_2O bath for 5 minutes, and slowly cooled to room temperature. 1 μl 1M $MgSO_4$, 3 μl dNTP mix (as for the first strand), 2 μl H_2O, and 2 μl of Klenow enzyme are then added in order to tube A (final volume = 60 μl). 4 μl of this reaction mix are immediately transferred to tube B. Both tubes are then incubated at 15°C for 4 hours.

T4 DNA polymerase is used to ensure that the termini of the double-stranded cDNA are even. 8 μl buffer, 2 μl dNTP mix, 4 μl BSA, 4 μl H_2O, and 1 μl enzyme are added directly to tube A; 1 μl buffer, 1 μl BSA, 2 μl H_2O, and 1 μl enzyme are added to tube B, and both tubes are incubated at 37°C for 15 minutes. The reaction is halted with 0.1M EDTA (5 μl, tube A; 1 μl, tube B). 1 μl is removed from tube B and diluted to 10 μl (1 μl for PEI cellulose chromatography, 1 μl for TCA precipitation and 1 μl for total dpm). Recovery is calculated as previously described for first strand synthesis (specific activity = 51.8 dpm/ng).

C. Modification of cDNA

Solutions

1. 10X linker-kinase (L-K) buffer (0.5 M Tris·HCl, pH 7.6, 0.1M $MgCl_2$)
2. EcoRI linkers (1 U/107 µl H_2O = 10 nmoles/ml, stored at -20°C.)
3. 10X methylase buffer (1.0M Tris·HCl, pH 8.0, 0.05 M EDTA)
4. S-adenosylmethionine (SAM; 2 x 10^{-4}M; stored in 20 µl portions at -20°C)
5. yeast tRNA (10 mg/ml, filtered and stored in 10 µl portions at -20°C)

Procedure

The double-stranded DNA from the previous reaction is extracted with phenol and precipitated as described earlier (10 µl yeast tRNA as carrier, 1/2 volume of 7.5M NH_4Ac, 2.5 volumes of EtOH). The sample is left overnight at 0-4°C, centifuged for 20 minutes in the microfuge, and air dried.

The cDNA is subsequently protected from EcoRI digestion by methylation of EcoRI sites with EcoRI methylase. 14 µl H_2O, 2 µl 10x methylation buffer, 2 µl SAM, 1µl BSA, and 1 µl enzyme are combined and incubated at 37°C for 1 hour. Enzyme activity is terminated by extracting with phenol in a 70° bath for 15 minutes. The sample is precipitated as described above with NH_4Ac and EtOH, dried under vacuum, and resuspended in 9 µl H_2O.

EcoRI linkers are phosphorylated utilizing T4 polynucleotide kinase. 10 µl H_2O, 1 µl 0.1M ATP, 2 µl EcoRI linkers, 2 µl L-K buffer, 2 µl BSA, 2 µl DTT, and 1 µl enzyme are combined. Following an incubation at 37°C for one hour, the sample is diluted to 10 µl with TE_2 and stored at -20°C. Phosphorylated linkers are then ligated to the cDNA as follows: 9 µl cDNA (see above), 2 µl L-K buffer, 2 µl BSA, 2 µl DTT, 2 µl ATP, 1-2 µl

T4 ligase, and 2 µl kinased linkers are combined and incubated at 15°C for 12-24 hours. The sample is deproteinized with phenol and precipitated with 4M NH_4Ac and EtOH as described previously.

In order to generate single EcoRI cohesive termini, the cDNA is then incubated with 3 µl EcoRI buffer, 1 µl BSA, 5 µl H_2O, and 1 µl (10U) EcoRI at 37°C for 45 minutes. The incubation is then continued for 45 minutes with an additional µl of EcoRI. The reaction is stopped with 5 µl 0.1M EDTA. After heat inactivation at 70°C for 5 minutes, a Select-2L spin column is used to eliminate linkers following the manufacturer protocol. The DNA in the 100 µl eluate is precipitated with 2 µl tRNA, 50 µl 7.5M NH_4Ac, and 250 µl EtOH (15 minutes, dry ice-EtOH bath), washed with 100 µl 80% EtOH, and reprecipitated (15 minutes, dry ice-EtOH bath). The cDNA is then resuspend in 5 µl TE and 1 µl is removed and diluted to 7 µl. 1 µl of this (1/35 dilution) is counted to determine the quantity of cDNA recovered (see reaction 1) and 6 µl is returned to the original tube. The sample is then adjusted to 50 ng cDNA/10 µl volume.

D. Ligation into λgt10 (Legersky and Robertson, 1985; Dugaiczyk *et al.*, 1975)

Procedure

5.0 µl L-K buffer, 5.0 µl ATP, 5.0 µl DTT and 5.0 µl BSA are combined to produce the ligation buffer. Ligation buffer, cDNA (50 ng/µl), control insert (200 ng/µl), uncut λgt10 (100 ng/µl), and λgt10 arms (0.5 µg/µl) are then added to 0.5 ml microfuge tubes in the volumes (µl) indicated below. All tubes are incubated at 15°C for 12-16 hours.

tube	ligation buffer	control insert	λ	λ arms	cDNA	ligase	final vol.
A	4	-	-	1	-	1	10
B	4	1	-	-	-	1	10
C	4	-	1	0	-	1	10
D	8	-	-	8	2	2	20

E. Packaging

The packaging of λgt10 DNA (figure 2) followed the Gigapack Gold system protocol using one-half of tubes A-D from the ligation reaction. Following packaging, all samples are diluted to 0.5 ml with SM, ~10 µl $HCCl_3$ is added, and tubes are stored at 0-4°C. The phage are titered at 3 dilutions on both C600 and C600 *hfl*A bacterial strains as directed by Davis *et al.* (1976). [Anticipate 10^5 pfu/µg λ arms (tube A - background negative control); 10^7 pfu/µg λ arms+ test insert (tube B - ligation positive control); $2x10^8$ pfu/µg uncut λ (tube C - packaging positive control); 10^7 pfu/µg λ arms+ cDNA insert (Tube D - library)].

F. Amplification

Amplification is performed precisely as described by Maniatis *et al.*, (1982), p 293.

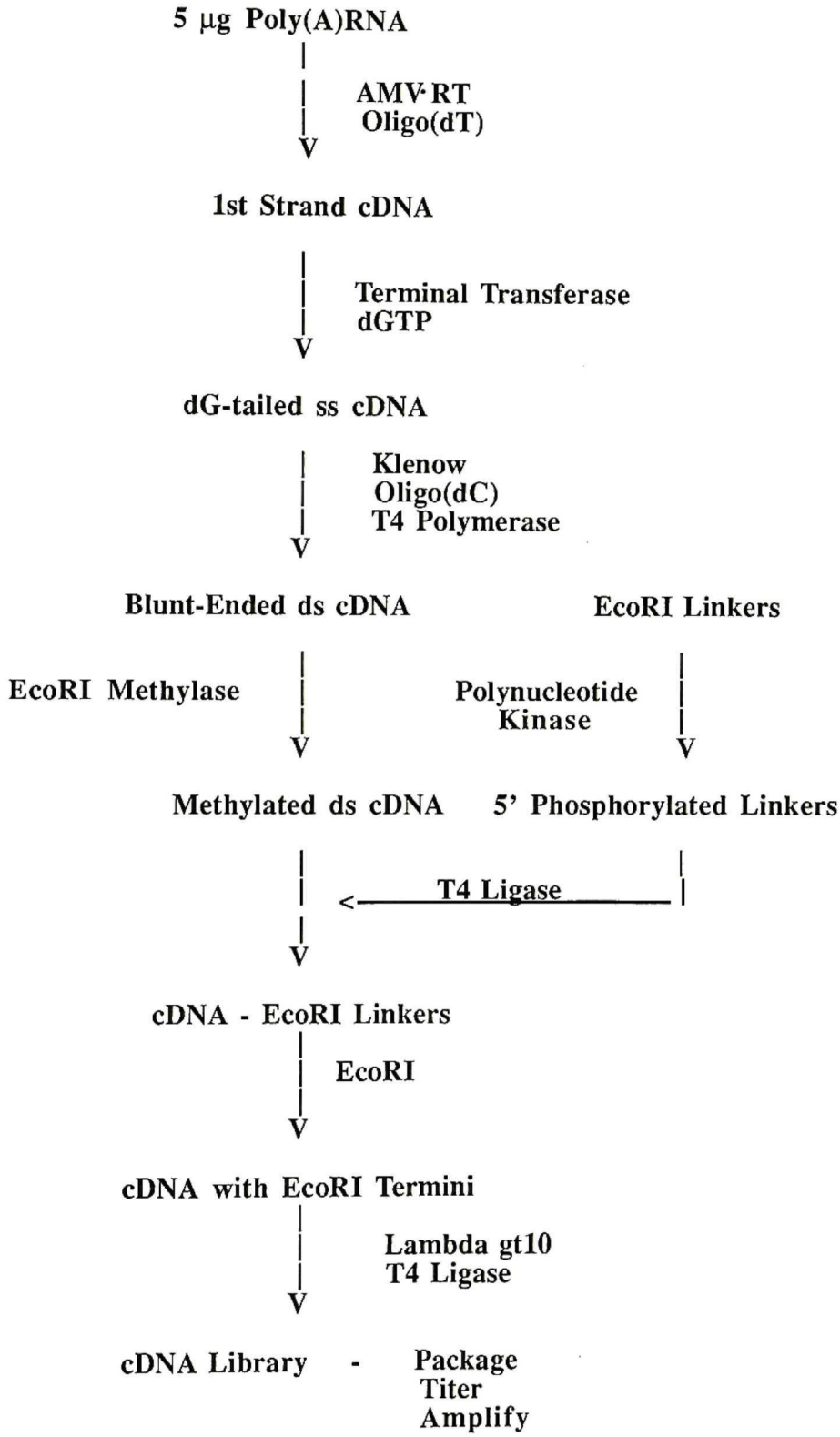


Figure 1. cDNA Synthesis

VI. Radiolabeling Procedures

A. Nick-Translation (Rigby *et al.*, 1977)

Solutions

1. Reagent A [0.2 mM dNTP mix (dGTP, dCTP, dTTP); 0.5M Tris·HCl, pH 7.5; 0.05M $MgCl_2$; 0.1M 2-mercaptoethanol; 100 µg/ml BSA; BRL nick-translation kit)
2. Reagent C (0.4 U/µl DNA polymerase I; 40 pg/µl DNase I; 0.005M Tris·HCl, pH 7.5; 0.005M 1M MgAc, pH 5.5; 0.001M 2-mercaptoethanol; 0.1M PMSF; 100 µg/ml BSA; 50% glycerol; BRL nick-translation kit)

Procedure

The DNA to be labeled (0.1-1 µg), 2 µl reagent A, 10 µl (100µCi) [α-^{32}P]dATP (400 Ci/mmol), and 2 µl reagent C are combined in a final volume of 20 µl. Following a 1 hour incubation at 15°C, the reaction is terminated with 5 µl of 0.5M EDTA and 75 µl TE_2. The volume is adjusted to 200 µl with 2X STE, 5 µl of phenol red is added to monitor removal of [^{32}P] nucleotide residues, and the DNA is recovered by centrifugation (3000 rpm in a clinical centrifuge for 4 minutes) through G50 sephadex spin columns. The DNA is precipitated with 100 µl 7.5M NH_4Ac and 600 µl ETOH and resuspended in 200 µl TE_2. The specific activity is determined by chromatography on PEI cellulose.

B. End-Labeling (T4 Polynucleotide Kinase; Sambrook *et al.*, 1990, p 10.59; Harrison and Zimmerman, 1986; Berkner and Folk, 1977)

The following reagents are combined in a 0.5 ml microfuge tube: 1-5 µl DNA (0.001 - 1 µg), 2 µl linker-kinase (L-K) buffer, 1 µl [γ-^{32}P]ATP, 2 µl BSA, 2 µl DTT, 1

µl T4 kinase, and H_2O to 20 µl final volume. Samples are incubated for 1 hour at 37°C. Unincorporated radionucleotides are removed using G50 sephadex spin columns as described above.

C. Labeling with Random Hexamer Priming (Feinberg and Vogelstein, 1984)

The DNA to be labeled is purified by electrophoresis on low melting agarose (in the case of restriction fragments). The fragment is isolated using GeneClean or, alternatively, is labeled directly by excising the the appropriate band and adding 3 ml of H_2O per gram of gel. This is heated at 100°C to melt the agarose and denature the DNA. This can then be stored at 0-4°C. For actual labeling, 1- 20 µl of DNA (0.05 - 0.5 µg) is brought to 31 µl with H_2O, denatured at 90°C for 15 minutes, and transferred to a 37°C water bath for 5 minutes. 10 µl of reagent mix (Pharmacia oligonucleotide labeling kit), 2 µl of BSA, 5 µl of [α-^{32}P]dCTP, and 2 µl of Klenow enzyme are added, and tubes are incubated for 2-16 hours at room temperature. The reaction is terminated with 20 µl of stop buffer (Pharmacia oligonucleotide labeling kit), 30 µl of TE_2 and 100 µl of 2X STE. 5 µl of phenol red is then added and DNA is eluted from G50 sephadex spin columns as previously described.

D. Run Off Transcription (Melton *et al.*, 1984)

Solutions

1. 1M spermidine (filtered and stored in 1 ml portions at -20°C)
2. 5X transcription buffer (0.2M Tris·HCl, pH 7.5 at 37°C; 0.03M $MgCl_2$; 0.01M spermidine; filtered and stored in 1 ml portions at -20°C)
3. SP6 RNA polymerase (20U/µl; Promega)
4. T4 RNA polymerase (20U/µl; Promega)

Procedure

The desired plasmid (pGEM3Z, pSP64/65, etc.) is linearized with an appropriate restriction enzyme. Complete digestion is verified by gel electrophoresis. 4 µl transcription buffer, 2 µl DTT, 1 µl RNasin, 1 µl each of 0.01M ATP, CTP, and GTP, 2.4 µl of 0.1 mM UTP, 1 µl (~1 µg) plasmid, 5 µl (50 µCi) [^{32}P]UTP, and 1 µl SP6 or T7 polymerase are combined in order. This mixture is incubated for 1 hour at 37°C and 1 µl of enzyme is then added for a second hour. Samples are treated with 1 unit (~1 µl) of RNase-free DNAse per µg starting DNA for 15 minutes at 37°C. The volume is adjusted to 100 µl with TE_1 and the sample is extracted with equal volumes of phenol:chloroform and chloroform. The phenol and $HCCl_3$ phases are re-extracted with TE_1 and the RNA is precipitated with 0.1 volumes of 4 M NaAc pH 6.0, 2.5 volumes of EtOH, and 1 µl glycogen as carrier. The radiolabeled RNA is resuspended in 100 µl 0.001M EDTA, pH 7.0, and 1 µl is applied to PEI cellulose TLC strips for determination of radioactivity in RNA.

E. Synthesis of Radiolabeled cDNA Probes (AMV·RT)

Synthesis of radiolabeled cDNA for screening of the cDNA library followed the basic procedure described for the first strand synthesis of cDNA (Section V) with the following exceptions: 200 µCi [α-^{32}P]dATP and 200 µCi [α-^{32}P]dCTP are added to the incubation mix, 2 µg poly(A) RNA is employed as the template, and 0.2 µg oligo(dT) serves as primer. The radiolabeled cDNA is recovered using G50 sephadex spin columns as described above. 1 µCi of [^{32}P]dCTP is generally used in experiments directed toward R_0t or C_0t analyses and in testing the recovery of single-stranded and double-stranded cDNA from hydroxylapatite columns. Other conditions are identical.

VI. Hybridization (Gal *et al.*, 1983; Sambrook *et al.*, 1990)

Solutions

1. 50X Denhardt's solution [1% (w:v Ficoll 400); 1% polyvinylpyrrolidone; 1% BSA; filtered and stored at -20°C]
2. 1M phosphate buffer, pH 7.0 (100 ml 2M NaH_2PO_4 + 150 ml 2M Na_2HPO_4 to 500 ml volume)
3. CHB (Church Hybridization Buffer; 1% BSA, 7% SDS, 0.5M phosphate buffer pH 7.0, 0.001M EDTA; 5 g BSA + 35 g SDS + 35.5 g Na_2HPO_4 + 1 ml 0.5M EDTA to pH 7.0 with ~5 ml phosphoric acid and brought to 500 ml final volume; filtered; Church and Gilbert, 1984))
4. Wash Buffer A (0.5% BSA, 5% SDS, 0.04M phosphate buffer pH 7.0, 0.001M EDTA; 2.5 g BSA + 25 g SDS + 20 ml 1M phosphate buffer pH 7.0 + 1 ml 0.5M EDTA to 500 ml volume)
5. Wash Buffer B (1% SDS, 0.04M phosphate buffer pH 7.0, 0.001M EDTA; 5 g SDS + 20 ml 1M phosphate buffer pH 7.0 + 1 ml 0.5M EDTA to 500 ml volume)
6. Hybridization Buffer I (50% formamide, 5X Denhardt's, 5X SSC, 10% dextran sulfate, 100 µg/ml DNA; 10 g dextran sulfate + 25 ml 20X SSC + 10 ml Denhardt's + 50 ml formamide + 1 ml salmon sperm DNA + 10 ml H_2O)
7. Hybridization buffer II [2X = 0.6M NaCl, 0.02M Hepes pH 7.0, 0.002M EDTA; deionized with AG-501-X8(D) mixed bed resin]
8. 10X S1 nuclease buffer (4M NaCl, 2M sodium acetate, pH 4.5; 0.05M $ZnSO_4$)
9. yeast tRNA (5 mg/ml)
10. salmon sperm DNA (10 mg/ml in TE_2; sheared and denatured)
11. Poly(uridylic) acid [poly(U); 10 mg/ml; stored at -20°C]

Procedures - Hybridization to Solid Supports (Meinkoth and Wahl, 1984; Minson and Darby, 1982)

Nytran Northern, Southern, dot, and slot blots (Reed and Mann, 1985) are prehybridized 5 to 10 minutes at 65°C in heat seal bags containing 10-20 ml of CHB. Hybridization is performed under the same conditions for 16 hours following addition of radiolabel (~ $5x10^6$ dpm/ml). Blots are washed 2 times in washing buffer A for 15 minutes at 65°C; 2-4 times in washing buffer B for 10 minutes at 65°C; and once in 0.2% SSC for 1 hour at 43°C. The blots are then air-dried on 3MM paper. Nytran blots can be stripped and rehybridized by heating to 90°C for 15 minutes in 0.1% SSC + 1% SDS.

Nitrocellulose filters for library screening (Thomas, 1980) are prehybridized 2-16 hours at 43°C in hybridization buffer I, allowing 1 ml per filter. Following addition of radiolabel (~10^6 dpm/ml buffer) and poly(uridylic) acid (1 µg/ml buffer), hybridization is continued for 16 hours. Filters are washed twice with 2X SSC + 0.2% SDS for 15 minutes at room temperature with gentle shaking. The filters are blotted on paper towels and rewashed twice in 0.2% SSC + 0.2% SDS for 15 minutes at 52°C. Filters are again blotted with 3MM paper and allowed to dry.

Procedures - Solution Hybridizations (Bishop *et al.*, 1974; Monahan *et al.*, 1976; Casey and Davidson, 1977)

For R_0t analysis of poly(A) RNA (Table 4), 30 µg E- poly(A) RNA is centrifuged (15,000 rpm, 15 minutes in a microfuge) and the pellet is resuspended in 25 µl 0.001M EDTA to yield concentrations of 1.2 µg/µl and 0.12 µg/µl. The RNA is distributed to six 0.5 ml microfuge tubes producing final incubation concentrations between 4 and 300 µg/ml in a 30 µl volume. 15 µl of hybridization buffer II is added to each tube along with 1 µl RNasin, 1 µl poly(uridylic) acid, sufficient yeast tRNA to produce a final total RNA concentration of 300 µg/ml (0-9 µl), and radiolabeled cDNA (typically 30,000 dpm/tube =

2 ng). The volume is adjusted to 30 µl with H_2O and tubes are sealed, boiled for 2 minutes, and quick-chilled on ice. The reaction mix is overlaid with a drop of mineral oil, resealed and incubated at 43°C. 6 µl samples are withdrawn at time points between 6 minutes and 32 hours and frozen on dry ice for S1 nuclease digestion. Control tubes include a time 0 sample, and a tube containing only yeast t-RNA (600 µg/ml) which is sampled at each time point to determine reannealing.

For R_0t analysis of the vitellogenin standard, 1 µg vitellogenin mRNA is resuspended in 10 µl EDTA to produce a concentration of 0.1 µg/µl. 1 µl is placed in tube 1 and 8 µl is placed in tube 2. 1 µl RNasin, 50 µl hybridization buffer II, 20 µl radiolabeled vitellogenin cDNA (40,000 dpm/tube = 6.0 ng) are then added, the reaction volumes are adjusted to 100 µl and the incubations are performed as described above for time periods between 10 seconds and 3 hours.

C_0t analysis of the amplified cDNA library is performed as follows: cDNA inserts are isolated from λ recombinants by EcoRI digestion and electrophoresis in low melting agarose (0.7% gels, 15 minutes). The area containing inserts is cut out, and the DNA is isolated using Geneclean as directed by the manufacturer. DNA from 4 gels is pooled, quantitated and adjusted to concentrations of 1.6 µg/µl and 0.16 µg/µl. Volumes of cDNA between 1 and 10 µl are delivered to 4 incubation tubes, producing incubation concentrations between 4 and 200 µg/ml. 20 µl hybridization buffer, 1 µl poly(U), and 2.5 µl radiolabeled cDNA (60,000 dpm = 4 ng) are added to each tube, the volume is adjusted to 40 µl, and samples are incubated as described above. 10 µl samples are withdrawn at times between 30 seconds and 3 days for S1 nuclease digestion.

Three concentrations of pGEM3Z plasmid (0.5, 1.5 and 5 µg/ml in a 50 µl reaction volume are utilized for C_0t analysis of the DNA standard. Linearized radiolabeled pGEM3Z (40,000 dpm = 20 ng) and 25 µl hybridization buffer are added to incubation tubes, the volume is adjusted to 50 µl, DNA is denatured, and tubes are incubated at 43°C as described previously. 10 µl samples are withdrawn at time points between 10 seconds and 4 hours for nuclease digestion.

S1 nuclease digestion (Sambrook *et al.*, 1990, p. 7.58) is performed as follows: 4 µl of each sample is incubated at 37° for 1.5 hours with 3 µl S1 nuclease buffer, 22 µl H_20,

and 1 µl S1 nuclease. The reaction is terminated with 5 µl BSA and 400 µl TCA. Samples are placed on ice for 15 minutes, applied to GF/A glass fiber filters mounted on a Millipore multisample vacuum apparatus, and washed 2x with 1 ml 10% TCA. Total hybridized counts are determined by precipitation of a sample which is not treated with S1 nuclease.

R_0t values are calculated and normalized to standard salt conditions using available conversion tables (Britten *et al.*, 1974; Pearson *et al.*, 1977). Data are expressed as percent of dpm hybridized and fit to the function $y = P[1-\exp(-0.693R_0t / R_0t_{1/2})]$ using the MLAB curve modeling function and the NIH DEC10 computer system. The MacIntosh Apple program Cricket Graph is utilized to graph data (x-axis = log R_0t; y-axis = percent hybridized).

TUBE	RNA (μg/ml)	RNA (moles nt /L)	TIME (seconds)	R_0t	LOG R_0t
1	4	1.23×10^{-5}	60- ?	7.38×10^{-4}	-3.13
//					
6	300	9.22×10^{-4}	? - 1.15×10^{5} (32 hr)	1.06×10^{2}	2.02

ASSAY - BUFFER
RADIOLABELED cDNA (2 ng)
POLY(A) RNA (1-600 μg/ml)

PROCEDURE - WITHDRAW SAMPLES AT VARIOUS TIMES
DIGEST WITH S1 NUCLEASE
ACID-PRECIPITABLE COUNTS = *cDNA HYBRIDIZED TO RNA

PLOT - PERCENT HYBRIDIZATION =

$$P\ [1\text{-}EXP\ (-0.693 R_0t\ /\ R_0t_{1/2})]$$

GRAPH - X-AXIS = LOG R_0t
Y-AXIS = PERCENT HYBRIDIZATION

Table 4. R_0t Procedure

* refers to radiolabeled cDNA.

VIII. Screening of the λgt10 Library (Benton and Davis, 1977; Woo, 1979)

Solutions

1. 0.2M NaOH:1.5M NaCl
2. 2X SSC:0.4M Tris·HCl, pH 7.4 (50 ml 20X SSC + 84 ml 2M Tris·HCl + 16 ml 2M Tris base to a final volume of 500 ml)
3. 2X SSC:0.2% SDS
4. 0.2% SSC:0.2% SDS
5. 0.8% agarose + 0.01M $MgCl_2$ (top agarose)
6. 2M phosphate buffer, pH 6.8 (prepared by mixing equal volumes of 2M NaH_2PO_4 and 2M Na_2HPO_4)
7. 0.06, 0.10, 0.12, 0.14, 0.4 M phosphate buffers, pH 6.8 (prepared by dilution of 2M phosphate buffer, pH 6.8)

Other Equipment:

1. BA85 nitrocellulose filters (Schleicher & Schuell)
2. Nitrocellulose marking pen (Schleicher & Schuell)
3. waterproof india ink + 18 gauge needle
4. radioactive marker + pen - (~1 μCi [^{32}P]; 0.2% coomassie blue; stored in a 1.5 ml microfuge tube in a lead shield)
5. nitrocellulose forceps
6. LB plates (The plates should be relatively dry; these are poured at least 2 days before use and allowed to stand at room temperature for about 8 hours prior to plating phage)

Procedures

A 25-30 ml culture of host bacteria is grown overnight in LB, transferred to a 50 ml conical tube, and centrifuged for 10 minutes at 3000 rpm in a clinical centrifuge. The

supernatant is discarded and the cells are resuspended in 10 ml 0.01M $MgSO_4$. This preparation can be stored at 0-4°C for about 1 week. After calculating the number of plates required to represent all genes in the library (estimating ~$5x10^3$ pfu/90 mm plate), 100 to 150 ml of bacterial suspension + 100 ml SM is distributed into a corresponding number of sterile culture tubes. A portion of the library is diluted with SM to yield the appropriate number of pfu/plate in a volume of 100 µl, and 100 µl of the phage are added to each culture tube. Tubes are gently agitated and incubated for 20 minutes at room temperature or at 37°C to adsorb the phage.

Approximately 4 ml top agarose/plate is melted and cooled to 60°C; in the meantime, LB plates are warmed in a 37°C incubator. 3-3.5 ml of top agarose is added to each culture tube, working 2 or 3 at a time. The tubes are mixed gently, poured over the surface of the plates, and allowed to sit at room temperature for about 20 minutes. Plates are incubated at 37°C for approximately 8 hours, monitoring phage growth carefully since it is desirable to have small plaques. Plates are transferred to 4°C at the optimal time, sealed with parafilm, and stored until screening.

Plates are chilled and transferred to room temperature (inverted) for 30 minutes to dry. Each plate and 2 corresponding filters are given an identification number. Duplicate filters are placed on the bacterial lawn (numbered side up) and left for about 20 minutes. Holes are keyed through the filters and into the top agar of each plate with an 18 gauge needle and waterproof ink. The filters are carefully peeled off the plates, dried on 3MM paper (numbered side down) for 30 minutes, and dipped sequentially (30-60 seconds/dip) in 100 ml of 0.2 M NaOH:1.5 M NaCl, 2X SSC:0.4 M Tris, and 2X SSC. Dipping solutions are changed every 25 filters. The filters are dried, numbered side down, on 3MM paper for about 1 hour, and are then baked in an envelope of 3MM paper in a vacuum oven (80°C, 2 hours) or in a standard oven (65°C, 3 hours).

The radiolabeled cDNA is synthesized essentially as described in Section VI with the exception that 2 µg of poly(A) RNA from estrogen-treated MCF-7 cells or from estrogen-deprived cells serves as the template, 0.2 µg oligo(dT) provides the primer, and a

total of 400 μCi of [α-^{32}P]dATP and [α-^{32}P]dCTP is present in the incubation. Following cDNA synthesis and removal of unincorporated nucleotides with a G50 sephadex spin column, the radiolabeled cDNAs are precipitated with 0.5 volumes of NH_4Ac and 2.5 volumes of EtOH (1-2 μg glycogen as carrier). The recovery of cDNA is determined on the basis of the specific activity of [^{32}P]deoxynucleotides in the original incubation mix. The radiolabeled cDNAs are each resuspended in 25 μl R_0t hybridization buffer (hybridization buffer II) with 7.5 μg of poly(A) RNA from estrogen-deprived MCF-7 cells and 0.2 μg poly(uridylic) acid (final volume = 50 μl). The hybridization is driven to a R_0t of 42 by incubating for 25 hours at 43°C. The radiolabeled cDNA remaining single-stranded is isolated by chromatography on hydroxylapatite as described below.

5 g of DNA grade hydroxylapatite is suspended in 0.06 M phosphate buffer pH 6.8 and washed 5 times with fresh buffer (shaking on an inverter for 3 minutes and centrifuging at 3000 rpm for 3 minutes in a clinical centrifuge). The slurry is stored at 4°C. Prior to chromatography, 0.75 ml of this slurry is boiled for 5 minutes in a 1.5 ml microfuge tube and cooled to 45°C. Radiolabeled cDNAs are diluted 10-fold with 0.06M phosphate (the final concentration of phosphate in the samples should not exceed 0.08M) to yield final volumes of 0.3-0.5 ml. The cDNA and 1 μl (10 μg) of sheared salmon sperm DNA are added to the hydroxylapatite, and allowed to incubate at 45°C for 10 minutes. The mixture is then poured into an econo-pac column (previously placed in a 65°C oven) and the eluate is collected. This eluate is reapplied to the column, again collected and the column is rinsed with 0.5 ml 0.06M and 0.5 ml 0.1M phosphate. These eluates are pooled as the run-through (discard) fraction. The column is then washed successively with 3 x 1 ml of 0.14M phosphate and 3 x 1 ml 0.4M phosphate buffers preheated to 65°C. 20 μl of each fraction is taken to monitor radioactivity. The profiles for elution of single-stranded and double stranded probes are shown in figure 2.

The radiolabeled cDNA (in 3 ml phosphate buffer) is concentrated by several extractions with 2-butanol (Maniatis *et al.*, 1982, p 463), to produce a final volume of 0.4 to 0.5 ml and is recovered by precipitation with EtOH and NH_4Ac as described above.

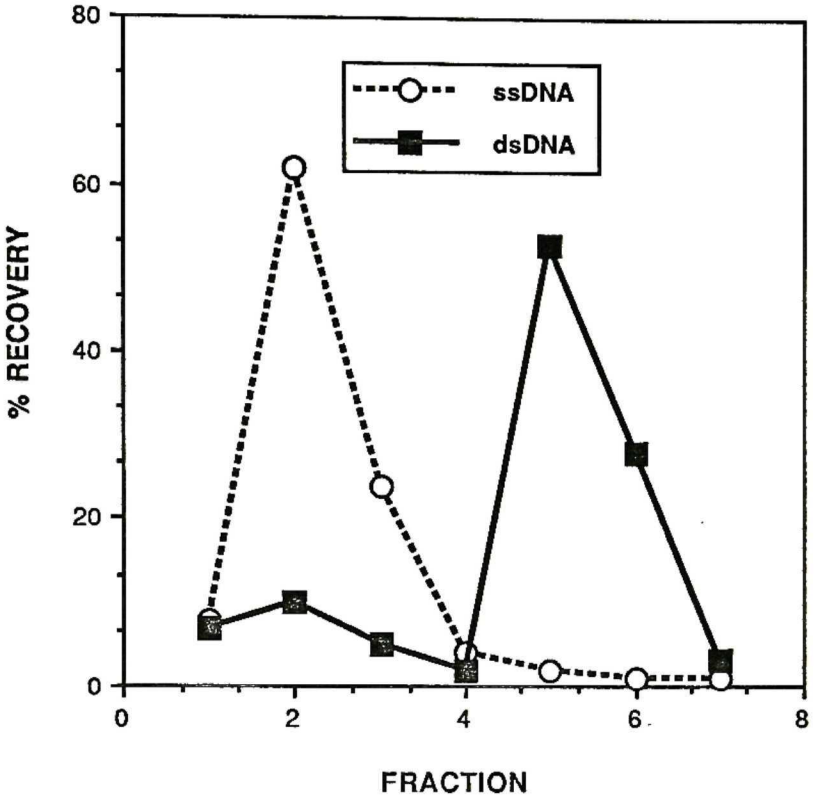
Radiolabeled probe is incubated with the filters (E+ probe with 1 set; E- probe with the second set) under hybridization conditions previously detailed in Section VII. After washing and air drying, radiolabeled marker is applied to the holes in the filters which match the master plate. The filters are taped to large sheets of 3MM paper, and the sheets are covered with plastic wrap. Autoradiography is performed at -70°C with an intensifying screen for 3 to 4 days (Laskey, 1980). Based on the match of autoradiograms to the master plates, plaques are isolated with sterile toothpicks into 0.5 ml SM. A drop of chloroform is added and the phage are stored in 1.5 ml sterile microfuge tubes at 4°C. The secondary and tertiary screens are performed essentially as described above with adsorption of phage from individual plaques to C600 *hfl*A host bacteria. However the phage are diluted to produce approximately 100 pfu/plate (one plaque is estimated to yield $5x10^6$ pfu). The procedures for filter lifts, preparation of probe, hybridization etc. are identical to those for the primary screen. The general schematic for selective screening of the cDNA library is shown in figure 3.

Figure 2. Elution of Single-Stranded and Double-Stranded DNA from Hydroxylapatite.

A. Elution Profile of Single-Stranded and Double-Stranded DNA Standards. Radiolabeled single-stranded cDNA was synthesized from E+ poly(A) RNA using AMV Reverse Transcriptase and 1 μCi [α-^{32}P]dCTP as previously described in Materials and Methods. Fraction 1 = discard (0.5 ml 0.06M phosphate + 0.5 ml 0.1M phosphate); Fractions 2-4 = single-stranded DNA (3 x 1 ml 0.14M phosphate); Fractions 5-7 = double-stranded DNA (3 x 1 ml 0.4M phosphate). 20,000 dpm [^{32}P]cDNA was first applied to the column and fractions collected as described previously. The column was rinsed thoroughly with 0.5 M phosphate until no radioactivity could be detected in the eluate. The column was then equilibrated with 3 x 1 ml of 0.06M phosphate. Subsequently 20,000 dpm of radiolabeled double-stranded DNA (prepared by nick-translation of the 1 kb DNA ladder or of 36B4) was applied and identical fractions were collected.

B. Elution Profile of cDNA Hybridized to Poly(A) RNA. 100,000 dpm of [^{32}P]cDNA was hybridized to a R_0t of 3.16 against E+ poly(A) RNA and applied to the column. Fractions were collected as described in A.

A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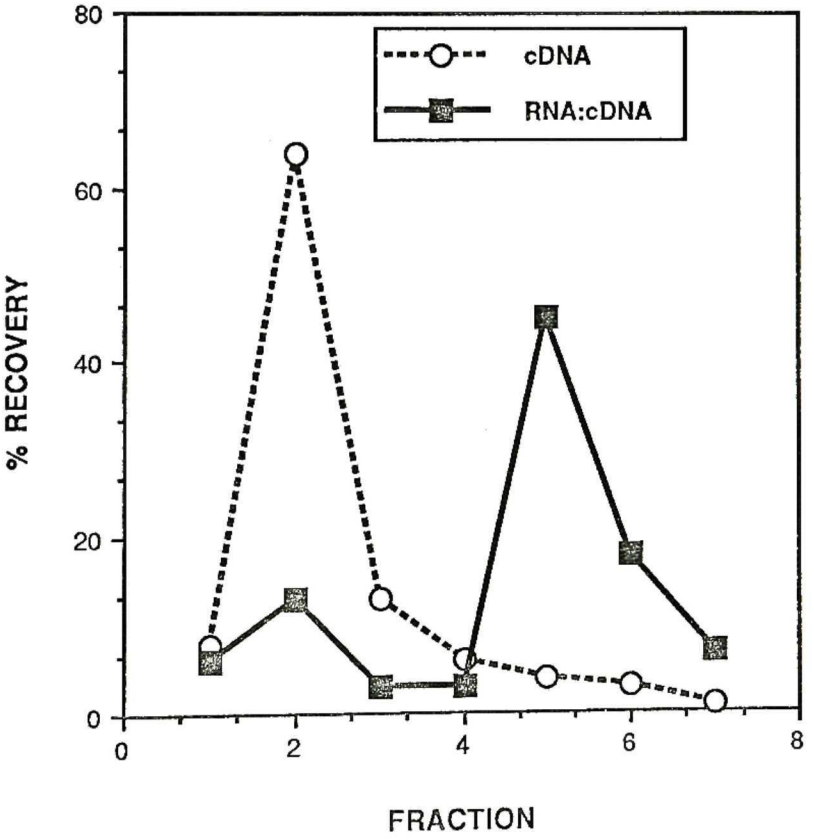
40
20
0
% RECOVERY
ssDNA
dsDNA
0
2
4
6
8
FRACTION

B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% RECOVERY
cDNA
RNA:cDNA
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FRACTION

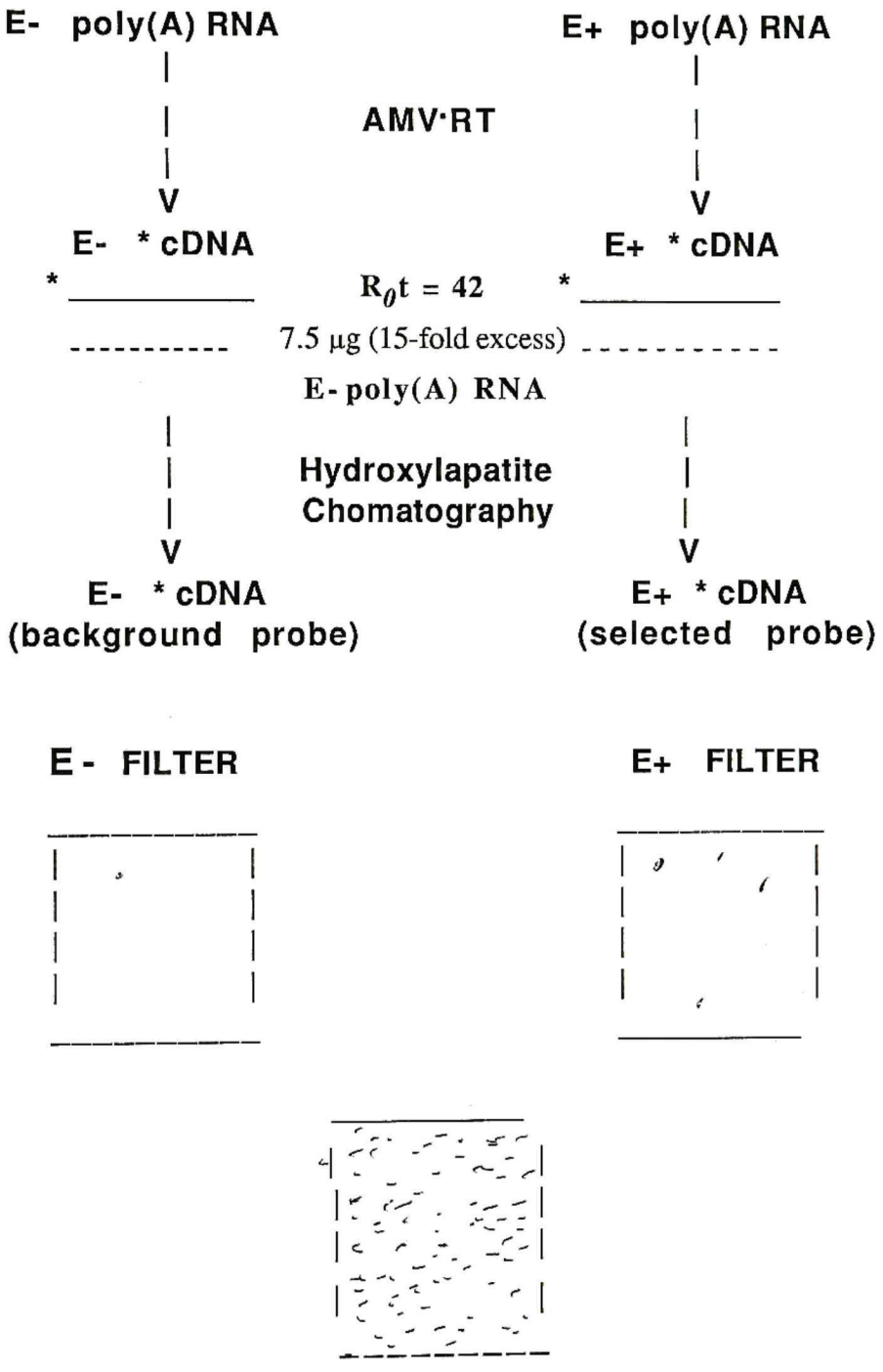


Figure 3. Selective Screening of the cDNA Library. * refers to [^{32}P] radiolabel.

IX. Sequencing (Williams *et al.*, 1986)

Solutions

1. Sequenase kit components

   Sequenase™ (modified T7 DNA polymerase)

   dGTP labeling mix (diluted 1:5 with H_2O; stored in 20 µl portions at -20°C)

   5X Sequenase buffer (0.2M Tris·HCl pH 7.5, 0.1M $MgCl_2$, 0.25M NaCl)

   Labeling Mix (7.5 mM dGTP, dCTP, CTP)

   Termination mixes (ddA = 80 mM each dNTP, 8 mM ddATP; ddT = 80 mM each dNTP, 8 mM ddTTP; ddC = 80 mM each dNTP, 8 mM ddCTP; ddG = 80 mM each dNTP, 8 mM ddGTP)

   Stop solution (95% formamide; 0.02M EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol)

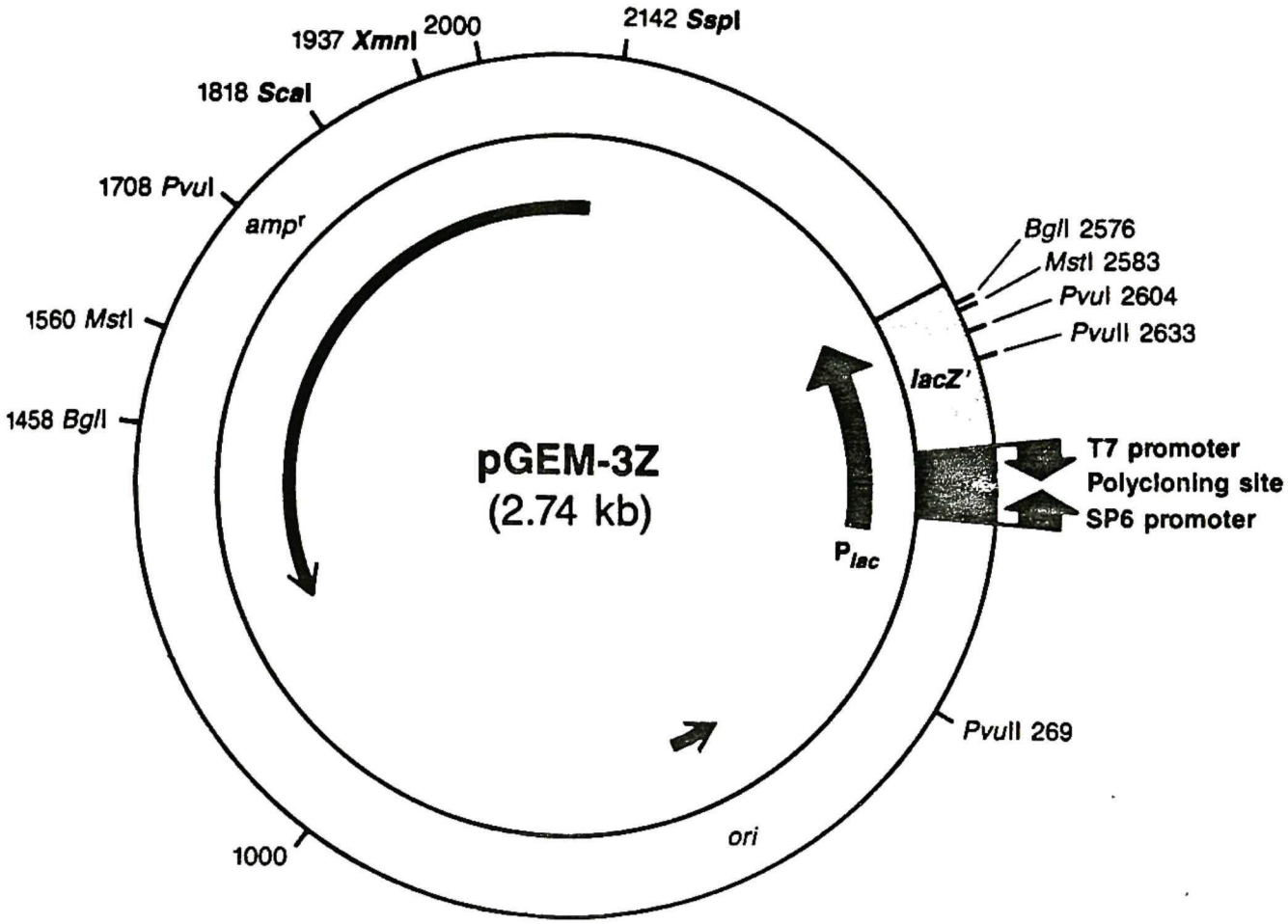
2. Fixing solution (5% acetic acid; 12% methanol)

Procedure

Sequencing of DNA is performed by the chain termination method of Sanger *et al.* (1977) employing modified T7 DNA polymerase (trade name Sequenase, Tabor and Richardson, 1987) and the Sequenase kit as detailed in the Sequenase technical manual. 4 µg of single-stranded plasmid (pGEM3Z, figure 4) is prepared by alkaline denaturation (20 µl plasmid in H_2O + 20 µl 0.8N NaOH for 10 minutes at 22°C; Zhang *et al.*, 1988). Following neutralization with 8 µl of 3M sodium acetate (pH 5.5) + 2 µl H_2O, the plasmid is precipitated by centrifugation (12,000g, 15 minutes) after adding 100 µl EtOH and incubating for 5 minutes on dry ice. The pellet is then washed with 100 µl 75% EtOH and recentrifuged. The DNA is air dried and resuspended in 7 µl H_20 with 2 µl of sequencing buffer and 1 µl (10 ng) of either SP6 promoter-primer or T7 promoter-primer. This mixture is annealed at 65°C for 2 minutes and slowly cooled to 22°C. Sequenase enzyme is diluted 1:8 (1 µl to 8 µl) immediately before use and kept on ice. 2 µl of diluted GTP

labeling mix is added to the tube along with 1 µl 0.1M DTT, 2 µl of diluted Sequenase enzyme and 1 µl [α-^{35}S]dATP. Following a 3 minute incubation at room temperature, 3.5 µl is distributed to 4 x 1.5 ml microfuge tubes (A, T, C, and G) which contain 2.5 µl of the respective ddATP, ddTTP, ddCCT and ddGTP termination mixes. The chain termination tubes are prewarmed to 37°C and are incubated for 2 minutes at 37°C following addition of the labeling mix. 4 µl of stop solution is then added to each tube and samples are stored at -20°C. Samples are heated to 70°C for 3 minutes prior to loading the sequencing gel.

An 8M urea, 6 % acrylamide wedge gel (5.7 g acrylamide, 0.3 g Bis acrylamide, 48 g urea, 10 ml 10X TBE, 40 ml H_2O, 50 µl 10% ammonium persulfate, 50 µl TEMED to 100 ml final volume) is prepared using the model STS-45 gel electrophoresis unit (IBI, New Haven, CN). TBE is placed in the buffer chambers, and gels are prerun for 1 hour at 1600-1800 V. After loading the gel, electrophoresisis is continued until the bromphenol blue dye just runs off the gel. The gel plate is then soaked for 15 minutes in fixing solution, and the gel is transferred to 3MM Whatman paper and covered in plastic wrap. Following drying, (gel dryer, 15 minutes, 80°C), the gel is ready for autoradiography (Kodak XAR-5 film, approximately 2-3 days).



Polycloning Sites

pGEM-3Z

T7 transcription start **SP6 transcription start**

GGGCGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGAGTATTC

EcoRI *SacI* *KpnI* *AvaI* *XmaI* *SmaI* *BamHI* *XbaI* *SalI* *AccI* *HincII* *PstI* *SphI* *HindIII*

pGEM-4Z

SP6 transcription start **T7 transcription start**

GAATACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGTCTCCC

EcoRI *SacI* *KpnI* *AvaI* *XmaI* *SmaI* *BamHI* *XbaI* *SalI* *AccI* *HincII* *PstI* *SphI* *HindIII*

Figure 4. pGEM-3Z

Figure reproduced from Maniatis *et al.*, 1990, p 1.17.

RESULTS

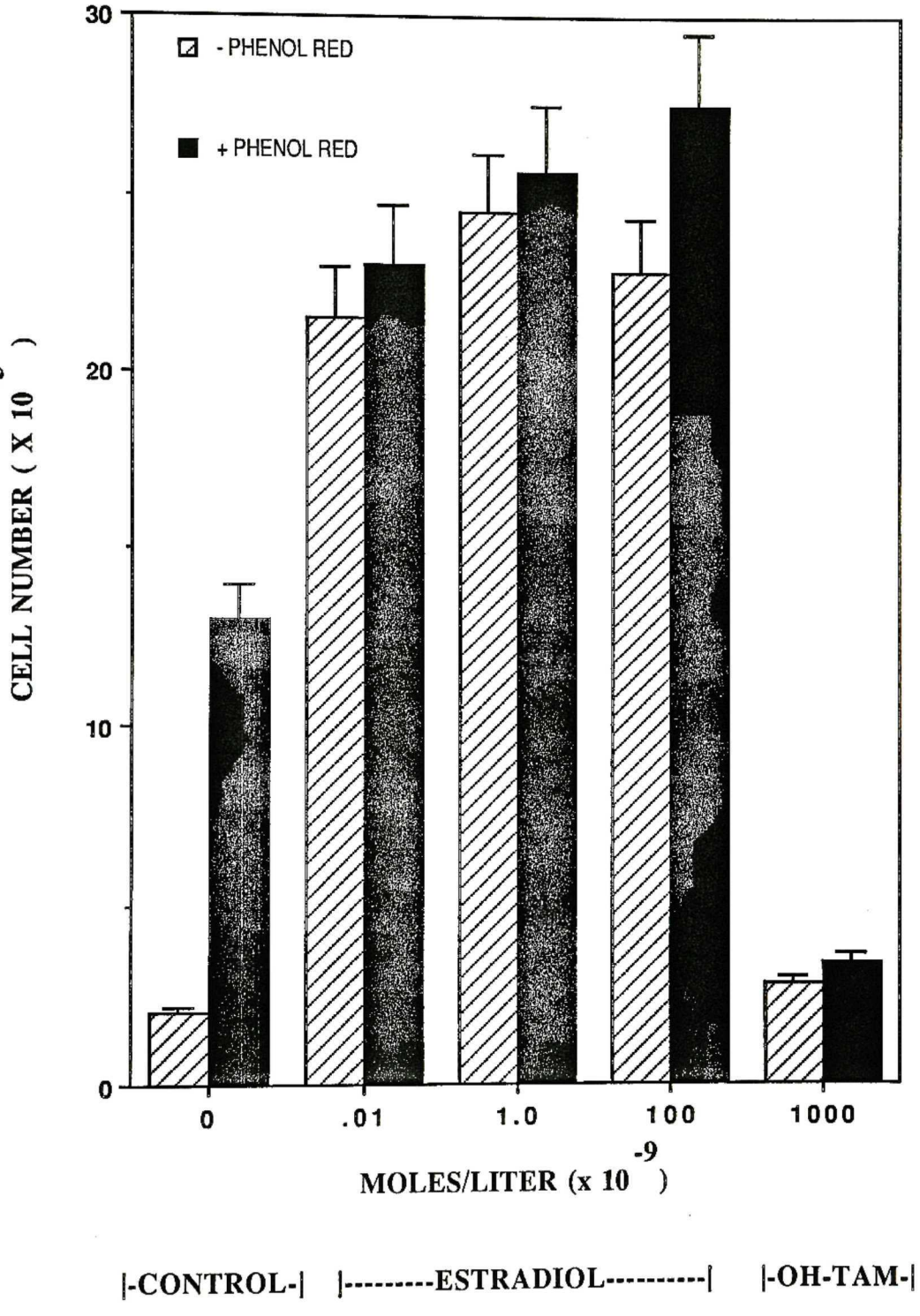
The first series of experiments in this investigation entailed verification that estrogen treatment constitutes the critical experimental determinant in regulating MCF-7 cell growth. Since phenol red has been a customary component of tissue culture media and is now believed to function as a weak estrogen, I first examined the effect of phenol red, estradiol (E_2), and the anti-estrogen 4-hydroxy-tamoxifen (OH-TAM) on MCF-7 cell proliferation (figure 5). Phenol red alone stimulated the growth of MCF-7 cells and this effect was prevented by the concomitant administration of OH-TAM. In addition the presence of phenol red masks the stimulatory effect of E_2 on cell growth. Clearly phenol red must be removed from the medium if MCF-7 cells are to be considered estrogen-deprived.

The effect of estradiol on the growth of MCF-7 cells over a seven day time period in the absence of phenol red is shown below (figure 6). Cells were maintained in medium lacking phenol red and supplemented with 2.5% charcoal-stripped serum and 10^{-9}M E_2. At time 0, the first experimental group (group 1, E+) received fresh medium supplemented with 10^{-9}M E_2. New E+ medium was supplied to the E+ group at days 1, 2, 3 , and 4. Also at time 0, the estrogen-supplemented medium in the second experimental group (group 2, E-) was replaced with medium lacking estradiol. The E- experimental group was again refed on days 1,2,3 and 4 with fresh E- medium. At day 4, however, one-half of the E- cells were refed with E+ medium (group 3, E-/E+). Cell counts were obtained at 24 hour intervals throughout the experiment. The doubling time of the E+ group over seven days was determined to be 1.86 days; doubling time of the E-/E+ group over three days was 1.76 days. The cell number of the E- cells remained constant. Under these conditions, it was concluded that estradiol comprised the only experimental variable regulating MCF-7 cell growth.

Nine independent experiments were subsequently conducted in order to isolate sufficient RNA from estrogen-deprived and estrogen-treated cells to permit a series of later studies. Cells were replicately plated in T150 tissue culture flasks and maintained in

Figure 5. Effect of Estradiol and Phenol Red on the Growth of MCF-7 Cells.

MCF-7 cells were maintained for 10 days in the presence or absence of phenol red ($5x10^{-5}$ M). Varying concentrations of estradiol (10^{-11}M, 10^{-9}M, 10^{-7}M)) or 10^{-6}M 4-hydroxy-tamoxifen (OH-TAM) were administered at 3 day intervals during the course of the experiment. Cell number was determined as described in Materials and Methods. Values are shown as the mean of 4 individual values +/- 1 SD.


30
20
10
0
- PHENOL RED
+ PHENOL RED
CELL NUMBER (X 10 -5)
0
.01
1.0
100
1000
MOLES/LITER (x 10 -9)
|-CONTROL-|
|---------ESTRADIOL----------|
|-OH-TAM-|

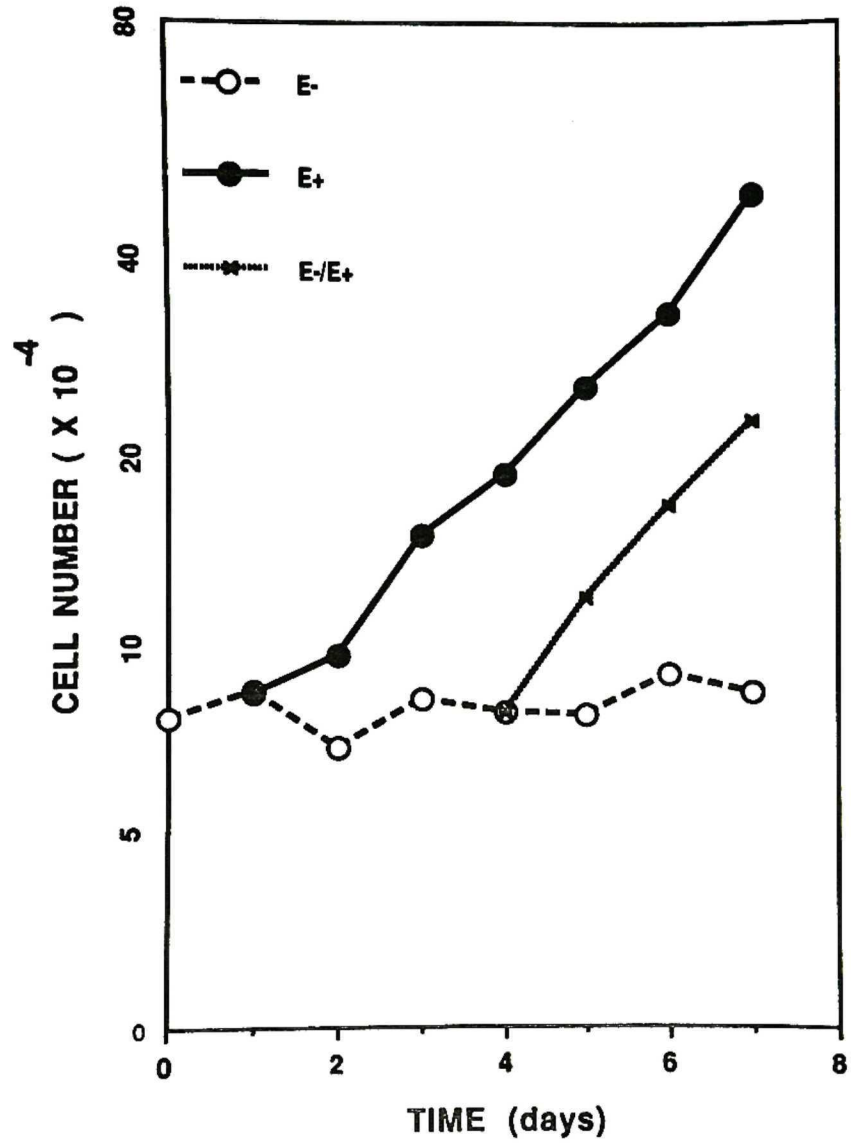


Figure 6. Effect of Estradiol on MCF-7 Cell Number.

Cells were maintained as described in Materials and Methods and in accordance with the experimental protocol delineated in Table 3. Identical numbers of cells were plated in replicate in 4 well Linbro dishes and plates were harvested at 24 hour intervals for determination of cell number (4 wells/experimental group) as previously described. Standard deviations ranged between 6 and 11%. E+ cells were continuously maintained in the presence of 10^{-9}M E_2 (fresh E+ medium was supplied at days 0, 1, 2, 3, and 4). E- cells were deprived of E_2 over the 7 day time course (fresh E- medium was supplied at day 0, 1, 2, 3, and 4). E-/E+ were similarly deprived of E_2 for days 1-4, and on day 4, cells received E+ medium.

medium lacking phenol red and supplemented with 2.5% charcoal-treated and sulfatase-treated calf serum and 10^{-9}M E_2. Fresh E+ medium was supplied at forty-eight hour intervals. At a density of approximately $4x10^5$ cells per flask and two days after the last treatment with estradiol, the E+ cells were collected. The E- cells were subsequently deprived of E_2 by three repeated daily medium changes, and were collected forty-eight hours after the last medium change. The exact protocol is detailed in table 3, p 64. Cell growth was measured through determination of cell number, of DNA, RNA, and protein content, and of [^{3}H]thymidine incorporation. In addition, total cellular RNA from the E+ and E- treatment groups in each experiment was tested for expression of individual mRNA species and for RNA integrity using Northern blots.

The mRNAs studied were pS2 and 36B4, kind gifts of Dr. P. Chambon, and gelsolin (A319, Mu319), generously placed at our disposal by Drs. C. Dieffenbach and R. Silverman. pS2 is known to be induced up to 100-fold by estradiol whereas 36B4 is not considered to be estrogen-regulated. Gelsolin mRNA levels are known to decrease under conditions which increase cell growth in other systems (Dieffenbach *et al.*, 1989). We thus hoped to verify that the mRNA preparations in each experiment reflected the hormonal regimen: i.e., that 36B4 mRNA expression was identical in the E- and E+ RNA preparations, that pS2 mRNA levels were greatly elevated in E+ RNA and extremely low in E- RNA samples, and that the amount of gelsolin mRNA was significantly lower in E+ RNA preparations than in E- preparations. 36B4, as expected, demonstrated no response to E_2 and pS2 (positive control) was dramatically elevated following two days of estrogen treatment (figure 7). In MCF-7 cells gelsolin mRNA levels fell to undetectable levels on treatment with estradiol, indicating that this mRNA is a suitable negative control in our experimental system. Densitometric analysis of autoradiograms of Northern blots routinely confirmed this pattern of expression. 36B4 hybridized to a single mRNA species of approximately 1.8 kb, pS2 identified a 0.6 kb mRNA, and gelsolin appeared to represent a single mRNA of approximately 2.2 kb. No degradation of RNA was apparent in any preparation.

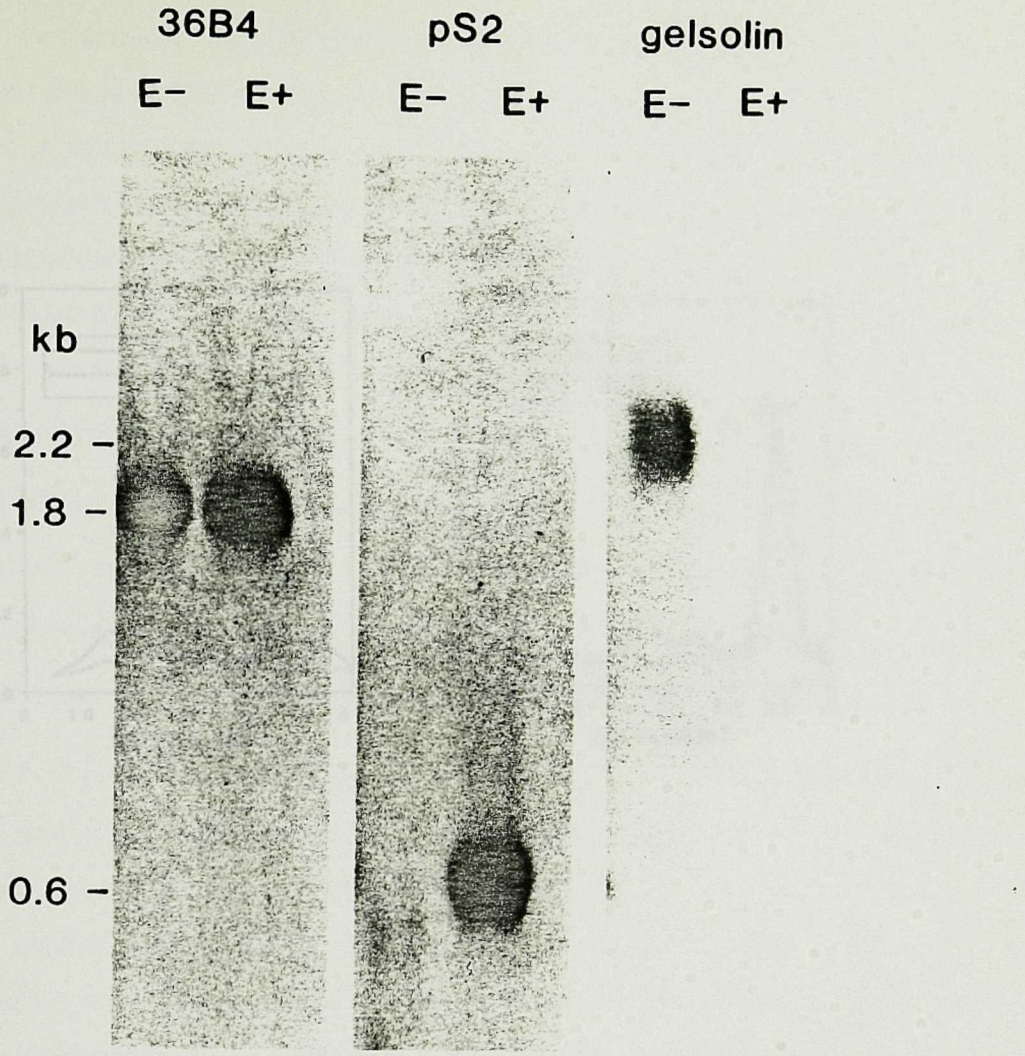


Figure 7. Northern Blots of Specific RNA Species from Estrogen-Treated and Estrogen-Deprived MCF-7 Cells.

Total RNA from E- and E+ MCF-7 cells was prepared and analyzed on glyoxal denaturing gels as described in Materials and Methods. Northern blots were hybridized with nick-translated or oligo-labeled 36B4, pS2, or gelsolin DNA. pS2 = 0.4 kb fragment of a 0.6 kb cDNA cloned into PBR322 and isolated by PstI digestion; gelsolin = 2.3 kb fragment obtained directly from Dr. C. Dieffenbach and Dr. R. Silverman; 36B4 = 2.2 kb fragment of a cDNA cloned into PBR322 and isolated by PstI digestion. Autoradiography was performed for varying periods of time (36B4, 12-16 hours; pS2, 12-16 hours; gelsolin 3-4 days).

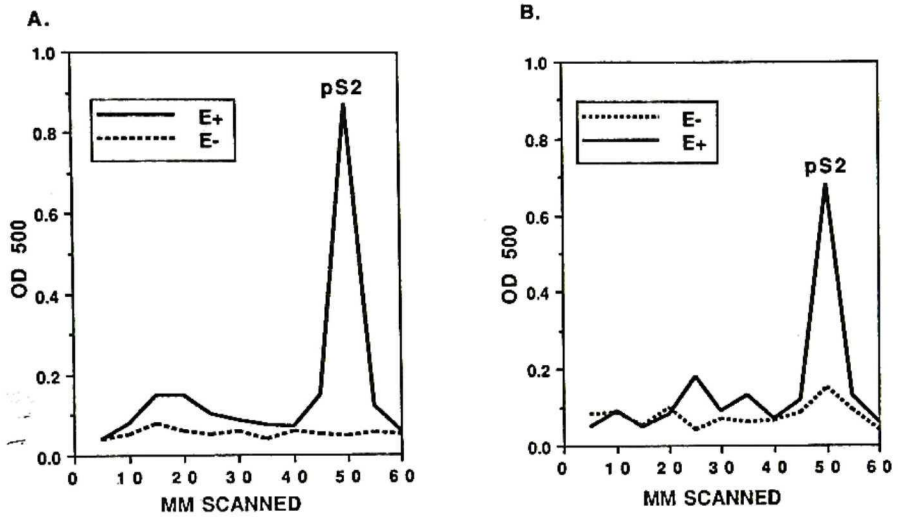


Figure 8. Densitometric Analysis of pS2 mRNA Expression.
A. Preparation 4. B. Preparation 7.

Densitometry was performed with the DU 8 spectrophotometer and gel scanning module. Peaks were identified and areas integrated using the internal computer programs of that module. Data were then redrawn using Cricket-Graph and the Apple MacIntosh II.

Two scans of Northern blots probed with pS2 are illustrated below (figure 8). Note that scan A is of preparation 4 and scan B is of preparation 7. A basal level of pS2 expression was detected in the E- poly(A) RNA from preparation 7 although E_2 did induce pS2 approximately 10-fold. In contrast pS2 was not detectable in the E- treatment group from preparation 4. The actual data on RNA preparations from each of the nine independent experiments is seen in table 5. The cell numbers, and the DNA, RNA and protein content of E+ and E- groups within a single experiment are similar. However, [^{3}H]thymidine incorporation characteristically increased from 10-100-fold in estrogen-treated cells, and the expression of pS2 also increased 20-100-fold. It was noted that the incorporation of [^{3}H]thymidine in E- cells from experiment 7 exceeded the average among all experiments by almost 4 standard deviations (0.58 vs. an average of 0.15). E_2 clearly still had a strong stimulatory effect, increasing [^{3}H]thymidine incorporation 10-fold. However, the high bsal level of both [^{3}H]thymidine incorporation and pS2 gene expression caused the E+ and E- RNA from experiment 7 to be excluded from the pool of poly(A) RNA used for cDNA library construction and screening.

Having isolated a large quantity of RNA from E- and E+ MCF-7 cells, the next set of experiments were directed toward construction of an E+ cDNA library in λgt10. The synthesis of cDNA, ligation and packaging are discussed in Materials and Methods, Section V. Phage from the primary library were titered on both C600 and C600 *hfl*A *E. coli* *E. coli* mutants designated *hfl* (high frequency of lysogeny) produce elevated amounts of the product of the λ CI gene (a repressor of λ gene transcription). Phage titered on such host strains are almost exclusively directed into the lysogenic pathway of development. The plaques seen on C600 thus include recombinant phage, whose CI function has been lost by insertion of a cDNA sequence, plus non-recombinant phage directed into lytic development at a defined probability. However, the plaques seen on C600*hfl*A should almost entirely reflect recombinant phage. Tube A (positive control for packaging) indicates the efficiency of λ *in vitro* packaging and infection (table 6). As expected, the *hfl* mutation greatly lowered the titer of non-recombinant uncut λ. Tube B (negative control) indicates the background produced by dephosphorylated λ fragments

	TREATMENT (E+/E-)	CELL # ($x10^{-5}$)	[^{3}H]thymidine Per Flask (dpm $x10^{-6}$)	DNA	PROTEIN	RNA	pS2
				($\mu g/10^6$cells)			(increase)
1.	+	4.13	1.32	8.0	1.47	35	+
	-	2.96	0 .11	12.0	1.28	29	
2.	+	7.20	1.06	12.2	1.63	85	+
	-	5.54	0.14	14.1	1.00	54	
3.	+	1.68	1.48	8.8	1.25	72	+
	-	1.50	0.09	10.4	1.00	54	
4.	+	6.05	1.74	12.5	1.03	68	+
	-	5.20	0.22	11.5	0.96	30	
5.	+	3.24	1.60	17.3	1.03	60	+
	-	1.36	0.03	24.1	1.05	74	
6.	+	3.94	1.73	14.2	1.62	44	+
	-	2.95	0.10	14.6	1.09	33	
7.*	+	9.06	5.68	10.2	1.03	37	+
	-	7.91	0.53	22.1	1.05	20	
8.	+	1.10	1.82	15.6	1.18	32	+
	-	0.80	0.05	9.0	0.90	44	
9.	+	4.63	1.48	13.8	1.47	54	+
	-	2.87	0.06	14.4	1.01	38	
192 E+ Flasks		4.55 +/- 2.72[a]	20.8-fold increase	11.8 +/- 3.20[a]	1280 +/- 300[a]	58 +/- 19[a]	20-100-fold increase
292 E- Flasks		3.57 +/- 2.28[a]		16.9 +/- 5.20[a]	1070 +/- 100[a]	34 +/- 12[a]	

Table 5. CRITERIA DEFINING E+ AND E- RNA PREPARATIONS.

[a] refers to the standard deviation between the individual experiments. Within an experiment the individual flasks were pooled and only one value was obtained for each parameter. Cells were treated and collected according to the protocol shown in table 3. The E+ and E- RNA preparations in experiment 7 (*) were not used in the final RNA pool used to construct the cDNA library, or to generate RNA for solution hybridizations.

	PACKAGING		LIGATION	
	(+ CONTROL)	(- CONTROL)	(+ CONTROL)	(LIBRARY)
TUBE	A	B	C	D
λgt10 Uncut	+ (100 ng)	-	-	-
λgt10 Arms	-	+ (500 ng)	+ (500 ng)	+ (4 μg)
Test Insert	-	-	+ (200 ng)	-
cDNA insert	-	-	-	+ (100 ng)
pfu / μg λ DNA				
C600	2×10^8	4×10^4	1.5×10^7	1.25×10^7
C600*hfl* A	5×10^5	ND	1.5×10^7	1.25×10^7

Final Titration of Primary Library = 6.85×10^6 pfu / μg λ

(3.42×10^7 pfu / μg cDNA)

Total Number of Recombinants = 6.4×10^6

Table 6. Ligation and Packaging of the λgt10 cDNA Library.

ND refers to not detectable.

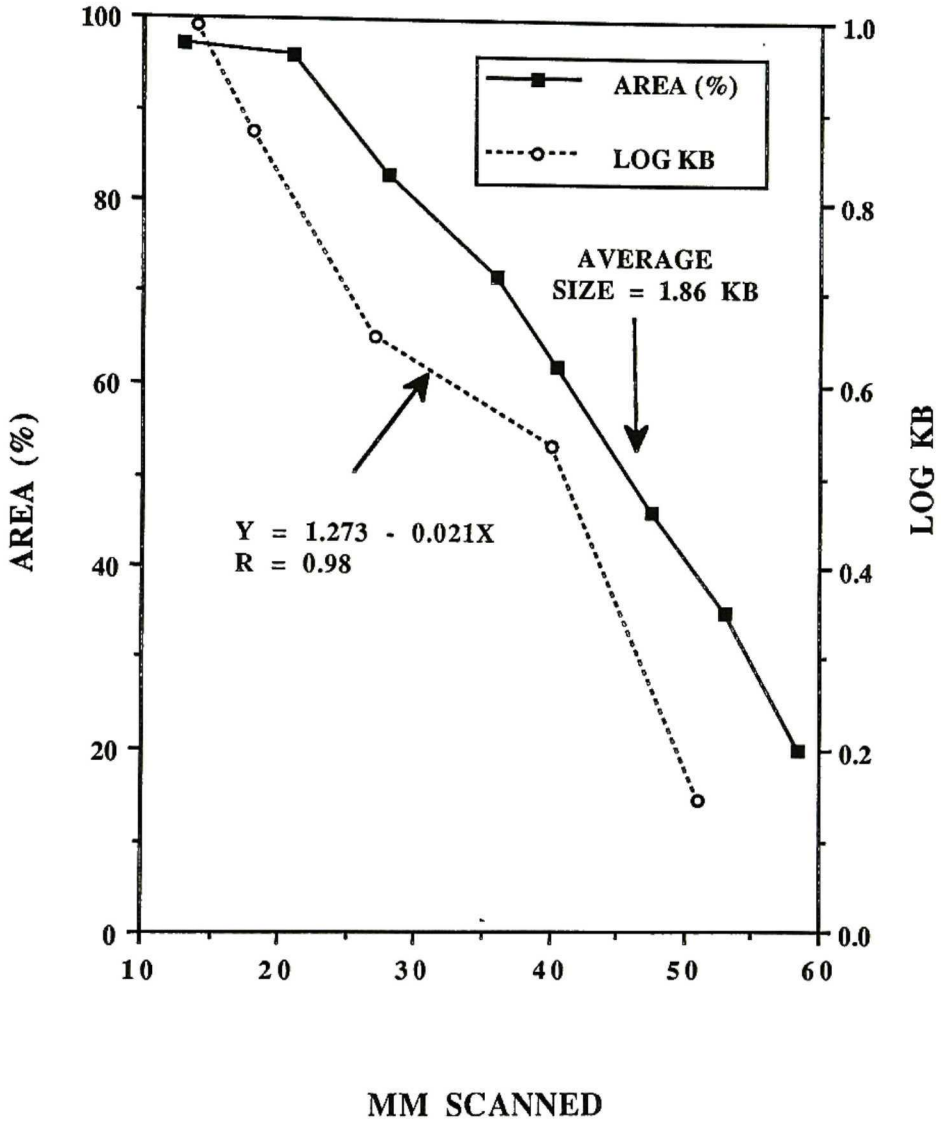
which are poor substrates for ligation and packaging. Here, the titer decreased by approximately three logs when compared with tube A, and the *hflA* mutation even further reduced the number of plaque-forming units (pfus). Tube C (positive control for ligation) tests the effect of ligation of a test insert on yield of packaged and infective λ DNA Successful ligation should also inactivate the product of the CI gene, yielding mostly recombinants. In this case, titer compared to tube B increased, but values on both C600 and C600 *hfl*A were similar. Tube D is essentially identical to tube C, but the inserts are the heterogeneous cDNAs synthesized from the E+ polyA) RNA template. The similarity in number of pfu in both host bacterial strains again indicated that most of the phage in the library were in fact recombinants. Further, the titer of the library is similar to that of the test insert (tube C), and the total number of recombinants (6.4×10^6) appeared to be sufficient to represent all the different RNA sequences in these cells (Bishop *et al.*, 1974).

It was then necessary to establish that the E+ cDNA library resembled the parent E+ poly(A) RNA population in terms of complexity and representation. We first determined the average molecular weight of the E+ poly(A) RNA by electrophoresis on denaturing gels, Northern blotting, and hybridization against radiolabeled E+ cDNA (weighted value) and against end-labeled oligo(dT) (unweighted value). The intensity of the signal obtained with radiolabeled cDNA reflects the size of that cDNA; however, an oligo(dT) probe will be of a single size, and only a limited number of molecules should hybridize per molecule of polyadenylated RNA. The weighted densitometric analysis is graphically represented in figure 9. The average molecular weight using radiolabeled cDNA was 1.86 kb; the unweighted value was determined to be 1.58.

Secondly, R_0t analyses were performed on E+ poly(A) RNA and a standard of known molecular weight. This procedure is discussed in the Hybridization section of Materials and Methods. A 1.6 kb cDNA for *Xenopus laevis* vitellogenin was selected as the standard. This DNA had been previously cloned into pSP64 (anti-sense RNA) and pSP65 (sense RNA) by A. Riegel (Riegel *et al.*, 1987). The R_0t analyses of the poly(A) RNA and of the vitellogenin standard are shown in figures 10A and 10B respectively. Three transition classes were evident in the E+ poly(A) RNA. In contrast, and as expected, the pure vitellogenin message manifested a single continuous curve.

Figure 9. Weighted Analysis of the Molecular Weight of E+ Poly(A) RNA from MCF-7 Cells.

2 μg of E+ poly(A) RNA was analyzed by electophoresis and Northern blotting onto Nytran. The Nytran blot was hybridized against 10^7 dpm of radiolabeled cDNA (100 ng) synthesized from 1 μg of poly(A) RNA template with AMV·RT as detailed in Materials and Methods. Large molecular weight RNA markers provided a standard curve. The DU 8 gel scanning module was employed to analyze the autoradiograms and the MacIntosh program Cricket Graph was used to present the data. The points representing % area were graphed against the left abcissa; the molecular weight standards were graphed against the right abcissa. The average molecular weight was calculated by drawing horizontally from the 50% value on the left y-axis to the corresponding point on the % area curve, and then extending a vertical line from that point to the ordinate. The corresponding ordinate value (X) was substituted into the equation $\mathbf{Y = 1.273 - 0.021X}$ derived from regression analysis of the $\log_{10}$ of the DNA standard sizes in kilobases (**Y**) vs. millimeters scanned (**X**).


AREA (%)
LOG KB
AVERAGE
SIZE = 1.86 KB
Y = 1.273 - 0.021X
R = 0.98
AREA (%)
LOG KB
MM SCANNED
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60
40
20
0
1.0
0.8
0.6
0.4
0.2
0.0
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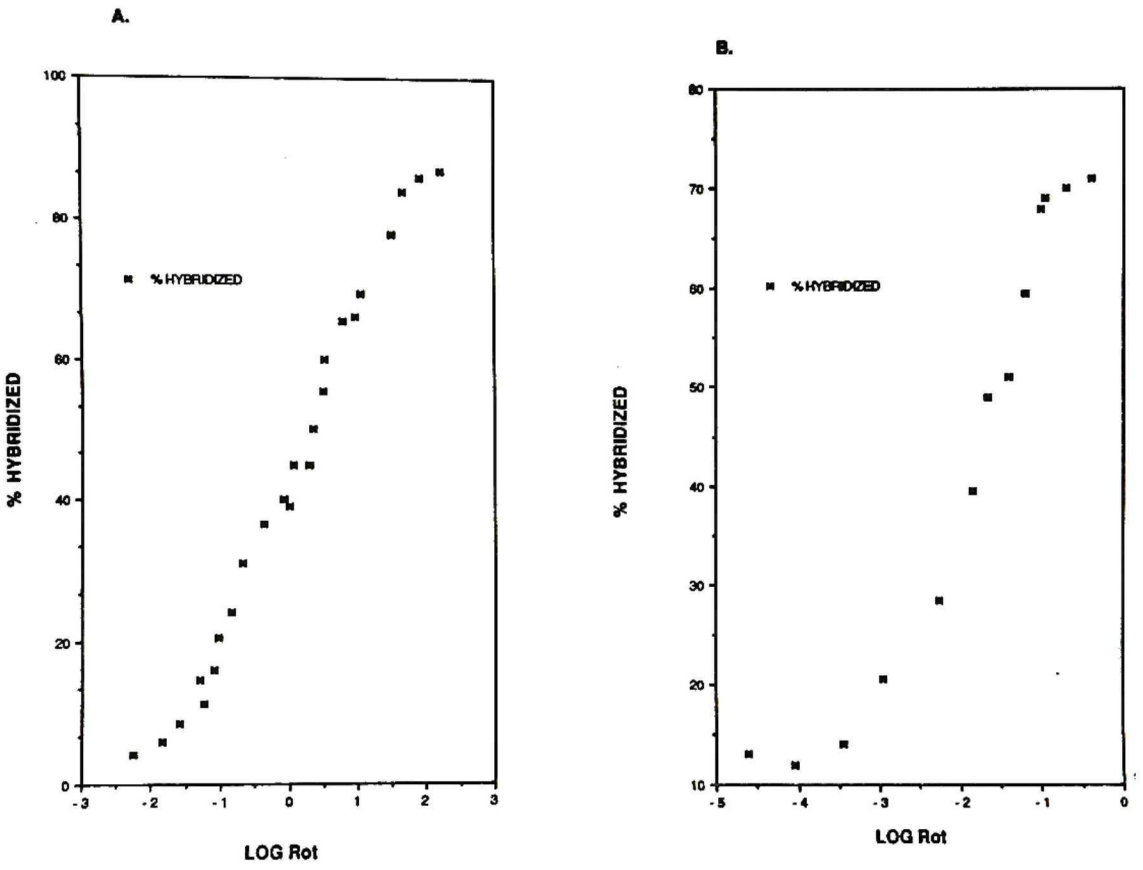


Figure 10. R_0t Analyses. A. Poly(A) RNA B. Vitellogenin mRNA

Assays were performed exactly as described in Materials and Methods (Section VII. Hybridization).

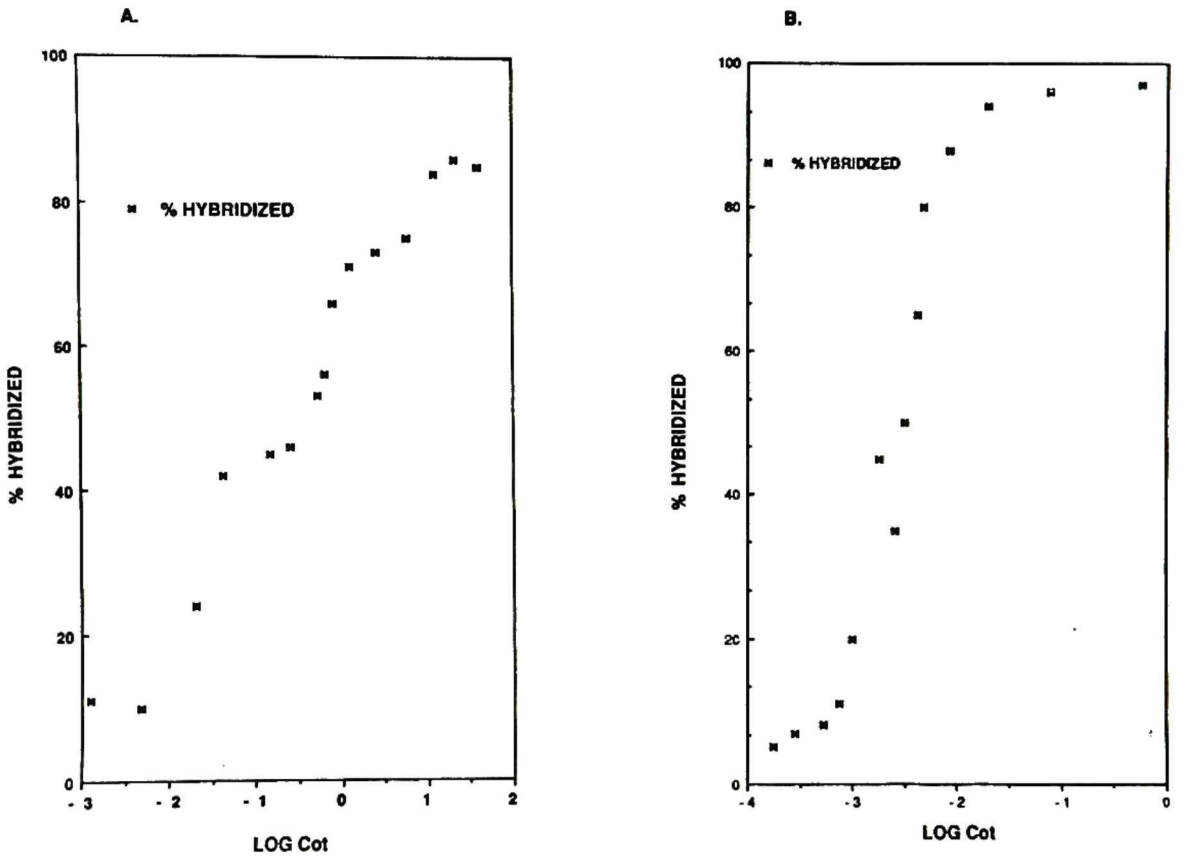


Figure 11. C_0t Analyses. A. cDNA Library Inserts B. pGEM3Z Standard

Analysis of the cDNA library was performed as described in Materials and Methods (Section VII. Hybridization). 2 μg of pGEM3Z was digested with HindIII, and 0.5 μg was then radiolabeled by nick-translation using 1 μCi [^{32}P]dATP (400 Ci/mmole).

Table 7. R_0t Analysis of Cellular Poly(A) RNA.

pSPxv10/65 (Riegel *et al.*, 1987) was linearized using HindIII, and run-off transcripts of mRNA were synthesized using SP6 polymerase as described in Materials and Methods. 250 ng of plasmid yielded 1 μg of vitellogenin mRNA. 1 μCi of [^{3}H]UTP was used to quantitate the amount of RNA produced (specific activity = 3.08×10^4 dpm/ng), and the size of the mRNA was verified by Northern blotting. Radiolabeled (*) vitellogenin cDNA was then synthesized using AMV-RT, 200 ng vitellogenin mRNA as template, 0.1 μg XBAI linkers as a primer, and [^{32}P]dCTP (1 μCi = 1.96×10^4 dpm/ng cDNA). 48 ng of vitellogenin *cDNA were recovered,and the size of the *cDNA was verified by electrophoresis on an alkaline agarose gel (1.2-1.6 kb). R_0t analysis of the vitellogenin standard was performed as described in Materials and Methods using 17,000 dpm of *cDNA per assay tube. *cDNA for R_0t analysis of cellular poly(A) RNA was synthesized using AMV-RT, 1 μg E+ poly(A)RNA as template, oligo(dT) as primer, and ~1μCi [^{32}P]dCTP as previously described. 30,000 dpm of *cDNA were added to each R_0t assay tube.

TRANSITION	P	$R_0t_{1/2}$	#DIFFERENT SEQUENCES	COPIES/CELL OF EACH SEQUENCE
I	0.36	0.263	26	7,684
II	0.29	9.54	923	174
III	0.22	93.3	9,240	13

CALCULATIONS:

1. Average size of cellular poly(A) RNA = 1.86 kb
2. $R_0t_{1/2}$ is determined by the number of molecules of an RNA species and the number of nucleotides per molecule (units for R_0t are nucleotides*sec/liter)
3. $R_0t_{1/2}$ of vitellogenin mRNA (1.6 kb) = 0.0087; therefore $R_0t_{1/2}$ of a 1.86 kb RNA = 0.0101
4. $R_0t_{1/2}$ poly(A) RNA / $R_0t_{1/2}$ standard = number of different poly(A) RNAs
5. 2 pg RNA/cell = 5.55×10^5 poly(A) molecules per cell
6. 5.55×10^5 molecules/cell x P / number of different sequences = number of sequences/cell)

The data derived from these experiments are shown in table 7. About twenty-six different mRNA species were found in the highly abundant class (Transition I). One thousand species comprised the moderately abundant class (Transition II) and the rare species (less than twenty molecules per cell; Transition III) numbered about ten thousand. These data were in general agreement with similar studies on human mammary cancer cells (Arya, 1982) as well as other cell types (Bishop *et al.*, 1974).

Parallel analyses were then conducted on the amplified cDNA library. Procedures are described in Materials and Methods. The C_0t analyses are shown in figure 11. Three transition classes were again evident in the cDNA library inserts whereas the pure DNA standard (pGEM3Z, 2.8 kb) demonstrated a single hybridization class. The data comparing the E+ cDNA library and the E+ poly(A) RNA template are summarized in table 8. The average molecular weight of the cDNA inserts was determined by electrophoresis of the EcoRI-digested amplified λ library on a 1% agarose gel, Southern blotting, and hybridization against radiolabeled cDNA and end-labeled oligo(dT) as described previously (data not shown). The decrease in average molecular weight in the cDNA undoubtedly reflected the presence of EcoRI sites within the inserts. However three classes of low, moderate, and high abundance messages were present in both the library and parent RNA populations, and the numbers of different sequences were very similar. It was concluded that the cDNA library did indeed mimic its E+ template and was suitable for the next experimental objective, screening and isolation of putative estrogen-regulated cDNA clones.

Hybridization kinetics had previously confirmed that the composition of the library (number of different species, molecular size, and frequency of individual species) indeed reflected that of the parent E+ poly(A) RNA template. The details of the library screening procedure are discussed in Materials and Methods. The isolation and plaque-purification of individual clones entailed three successive rounds of differential screening using radiolabeled cDNAs synthesized from either E- or E+ poly(A) RNA templates. Each cDNA was hybridized against excess E- poly(A) RNA to a R_0t value of 42. The cDNA remaining single-stranded was then isolated by chromatography on hydroxylapatite (table 9). On the basis of the previous R_0t and C_0t analyses, I calculated that screening of

	(weighted)	(unweighted)
$\bar{x}_{mw}$ RNA	1.86	1.58
$\bar{x}_{mw}$ cDNA insert	0.96	0.77
$R_0t_{1/2}$ Standard	1.01×10^{-2}	8.59×10^{-3}
$C_0t_{1/2}$ Standard	9.93×10^{-4}	7.96×10^{-4}

Transition	$R_0t_{1/2}$	#Sequences	$C_0t_{1/2}$	# Sequences
I	0.263	26/31	0.0214	21/27
II	9.540	923/1109	1.104	1115/1383
III	93.30	9,240/10,848	11.48	11,596/14,385

Table 8. Analysis of C_0t and R_0t Data.

		E+ (dpm *cDNA)	E-
Fraction 1	**Discard**	**2.00×10^6**	**2.00×10^6**
Fraction 2-4	**Single-Stranded**	**5.85×10^7**	**3.20×10^7**
Fraction 5-7	**Double-Stranded**	**3.16×10^8**	**2.30×10^8**

Table 9. Screening of the cDNA Library.

* refers to radiolabeled cDNA eluted from hydroxylapatite. Calculations: The number of different sequences in the low abundance class of cDNA library inserts = 11,596 and the fractional representation of that class (**P**) = 24%. The corrected number of different sequences (**n**) therefore = 48,316. Given a probability of 0.95 that any given clone will be represented in the recombinants screened, **N** (the number of clones which must be screened) = ln(1-P/ln(1-1/n) = **144,720**. Hybridization conditions and screening procedures have been previously descibed in Materials and Methods.

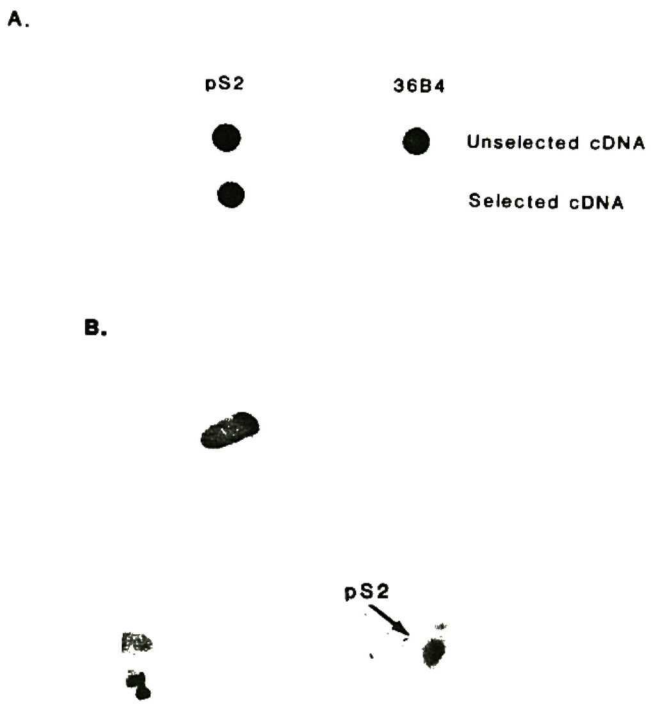


Figure 12. A. Removal of Sequences Common to E+ and E- cDNAs by Hybrid Selection.

200 ng of pS2 and 36B4 DNA was immobilized on a Nytran membrane as described in Materials and Methods (Section IV. DNA 'Dot Blots'). The blots were hybridized against approximately $5x10^7$ dpm of selected or unselected E+ cDNAs ($4x10^8$ dpm/μg). Autoradiography was performed for 2 hours at -70°C without an intensifying screen.

B. Presence of pS2 in the cDNA library.

10 μl of each of the 63 λ clones isolated on the first round of screening was dotted onto 2 nitrocellulose filters using a grid template to distinguish individual clones. The filter was then hybridized against approximately $5x10^7$ dpm of radiolabeled pS2 (upper panel). λ clone # 42 yielded a positive signal. The original nitrocellulose filter lift which was used in the primary screen and which contained clone 42 was then similarly hybridized against radiolabeled pS2 (bottom panel).

150,000 recombinants would permit a 95% chance of isolating a specific clone for an estrogen-regulated low abundance mRNA.

The above selection procedure ensured that approximately 80% of low abundance poly(A) RNA species, as well as virtually all moderate and high abundance poly(A) RNAs common to both E+ and E- RNA populations, would be eliminated from the radiolabeled cDNA. As previously noted, 36B4 is a moderately abundant mRNA which is not induced by estradiol. Unselected E+ radiolabeled cDNA hybridized against denatured 36B4 DNA produced a strong signal; no radioactive signal was seen on hybridization against a selected E+ radiolabeled cDNA (Figure 12A). In contrast, estrogen-inducible pS2 hybridized strongly against both unselected and selected radiolabeled cDNAs. Neither unselected nor selected E+ radiolabeled cDNA hybridized to denatured gelsolin DNA (data not shown).

The filters screened with an E- cDNA should primarily reflect a non-specific background, whereas duplicate filters screened with E+ cDNA should display a higher number of clones. Clones appearing differentially on either filter in the first round of screening were selected from the original plate and rescreened using a second set of selected radiolabeled cDNAs (figure 13). Nineteen clones were isolated on the third and final round of screening. Dot blots of denatured DNA from the λ clones were then hybridized with either an unselected E+ or E- cDNA probe. Nine of the nineteen were confirmed to be differentially expressed (data not shown).

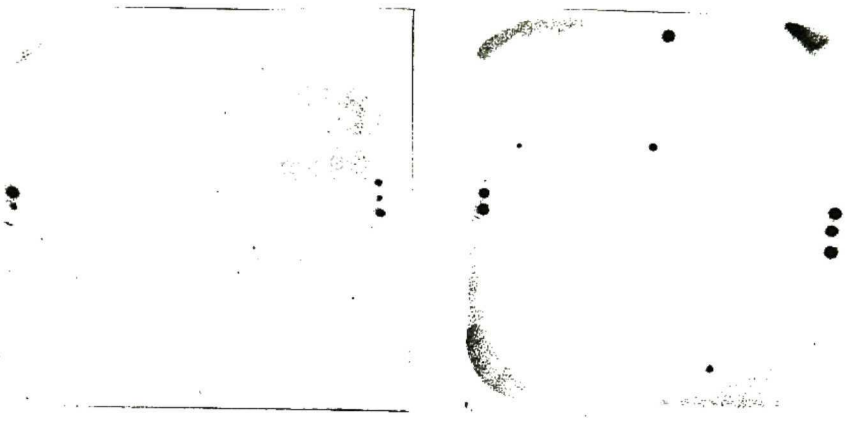
DNA isolated from each of these nine clones was subsequently digested with EcoRI and inserts were isolated by electrophoresis on low-melting agarose. Hybridization of all nine cloned cDNA inserts versus the corresponding radiolabeled inserts identified four distinct molecular species, one of which (pMT2) was represented six times (figure 14). None of the final nine clones proved to be pS2. However, one clone (#42) selected on the first round of screening was identified as pS2 (figure 12B). The hybridization of this particular clone to radiolabeled cDNA had not produced a strong signal and it had not been selected for examination in the second round of screening. In addition, one of the final nine cDNAs was subsequently identified as progesterone receptor (data not shown).

Figure 13. Primary, Secondary, and Tertiary Screening of the cDNA Library.

Representative autoradiograms of each round of screening are shown. Filters were hybridized against approximately 10^6 dpm of either E- or E+ radiolabeled cDNA per filter. Autoradiography was performed for 3 days at -70°C with intensifying screens.

PRIMARY

E- E+



SECONDARY

E- E+

TERTIARY

E- E+

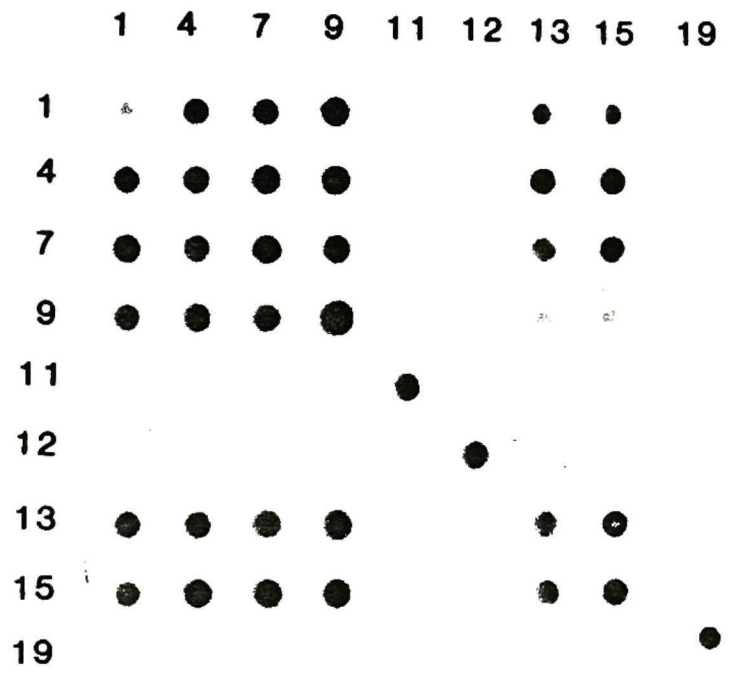


Figure 14. Matrix Hybridization of Selected Lambda Clones.

Denatured insert cDNA (~ 50 ng) from each of the final nine λ clones was immobilized onto a Nytran membrane using the Minifold dot blotting apparatus as previously described. Horizontal strips containing DNA from each clone were cut and each strip was hybridized against the radiolabeled cDNA insert (~10^7 dpm) indicated vertically. Strips were then reassembled for autoradiography.

1. PRIMARY SCREEN	**150,000**
2. SECONDARY SCREEN	**63**
3. TERTIARY SCREEN	**19**
4. MATRIX DOT BLOTS	
a) pS2 (secondary)	1 (clone 42)
b) E+/E- (tertiary)	9
c) oligo(dT)	17
d) PgR	1 (pMT1)

5. CROSSMATCH OF 9 FINAL CLONES - 4 DISTINCT SPECIES

	RNA	cDNA	Northern
pMT1	4.5; 5.2 kb	1.4 kb	E+
pMT2	11 kb	7.0 kb	E+
pMT3	1.7 kb	1.2 kb	E+
pMT4	2.6 kb	2.6 kb	E+

Table 10. Estrogen-Regulated Clones.

E+ refers to an increase in mRNA levels as detected on a Northern blot.

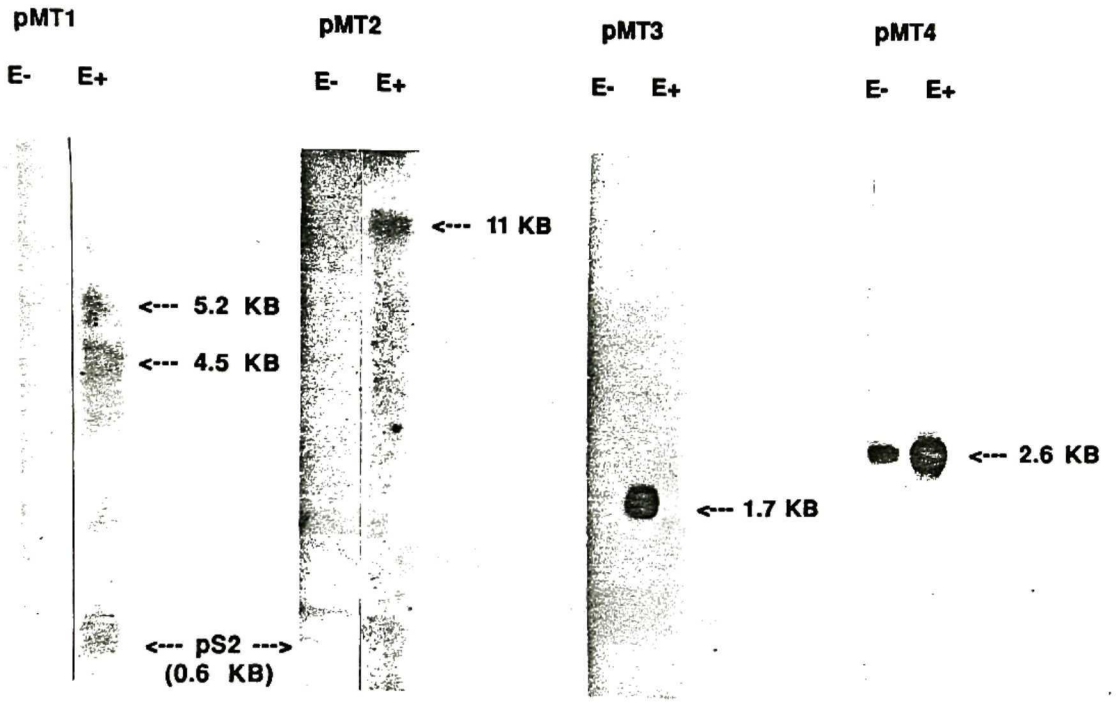


Figure 15. Northern Blots of Estrogen-Regulated Clones.

1 - 5 μg of poly(A) RNA from E- and E+ MCF-7 cells was analyzed as previously described. Autoradiography was performed for varying time periods (pMT1, 6 days; pMT2, 10 days; pMT3, 1 day; pMT4, 1 day).

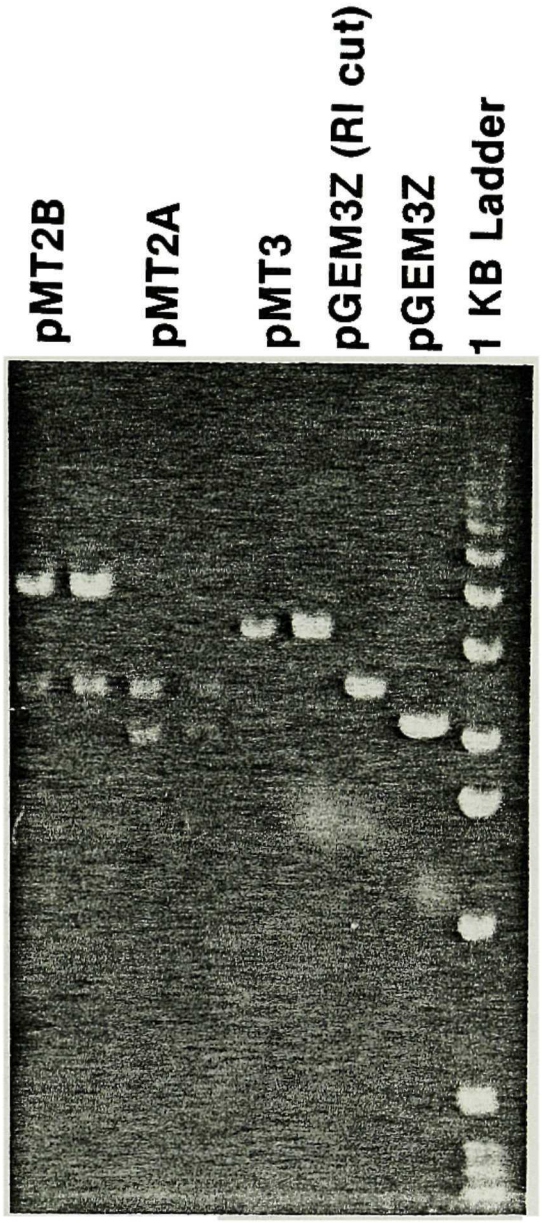


Figure 16. Subcloning of pMT2.

λ clones 12 and 13 were digested with EcoRI and the corresponding cDNA inserts (pMT3; pMT2A and pMT2B) were isolated by electrophoresis in 1% low melting agarose gels. DNA was prepared using GeneClean as specified by the manufacturer and inserts were ligated into the EcoRI digested vector pGEM3Z. The ligated DNA was then subcloned into HB101 competent cells according to the manufacturer protocol, and transformed colonies were isolated. DNA was isolated from plasmid minipreparations as described in Materials and Methods, digested with EcoRI, and analyzed on 1% agarose gels. In this experiment, pMT3 proved refractory to EcoRI digestion.

The data on the screening process and the identification of these estrogen-regulated clones are summarized in table 10. Northern blots of poly(A) RNA are shown in figure 15. pMT1 (progesterone receptor) appeared as two RNA species of approximately 4.5 and 5.2 kb. pMT2, pMT3, and pMT4 hybridized to single mRNAs of approximately 11, 1.7, and 2.6 kb respectively. All four were induced after three days of estrogen-treatment. pMT2 was subsequently subcloned into *E. coli* HB101 cells as two independent EcoRI fragments of 4.5 (pMT2B) and 2.2 kb (pMT2A) in the vector pGEM3Z (figure 16). The biological regulation of pMT2 was then examined in greater detail.

In the succeeding experiments MCF-7 cells were maintained in phenol red-free medium supplemented with 10^{-9} M estradiol and serum as described previously. Cells were then deprived of estradiol by four successive changes of estrogen-free medium at twenty-four hour intervals (day 0, 1, 2, 3). On day 4, cells were supplied with fresh medium with or without hormones (estradiol, 4-hydroxy-tamoxifen) as indicated in the particular experiment. Cells were collected as previously described in Materials and Methods and total cytoplasmic RNA was prepared according to Papavasiliou *et al.* (1986) for analysis on slot blots. All blots were hybridized against 36B4 (normalization of data), pS2 (positive control), gelsolin (negative control), and pMT2.

The first of these experiments demonstrated the effect of 10^{-9}M estradiol over a four day time course (figure 17). Both pS2 and pMT2 were induced after twenty-four hours and remained elevated. pMT2 mRNA could not be detected at time zero (no estradiol). In contrast the gelsolin mRNA was present at time zero and disappeared within twenty-four hours.

Varying concentrations of estradiol were then administered to MCF-7 cells in order to demonstrate that levels of estradiol which bind to estrogen receptor ($K_D = \sim 10^{-10}$M) also produce specific changes in gene expression. Results are shown in figures 18A ([^{3}H]thymidine incorporation) and 18B (mRNA expression). Cells were collected twenty-four hours after estrogen treatment. pS2 was induced by 10^{-12}M E_2 and induction was maximal at 10^{-10}M. Levels declined at 10^{-7}M and mRNA was not detectable at 10^{-6}M. In

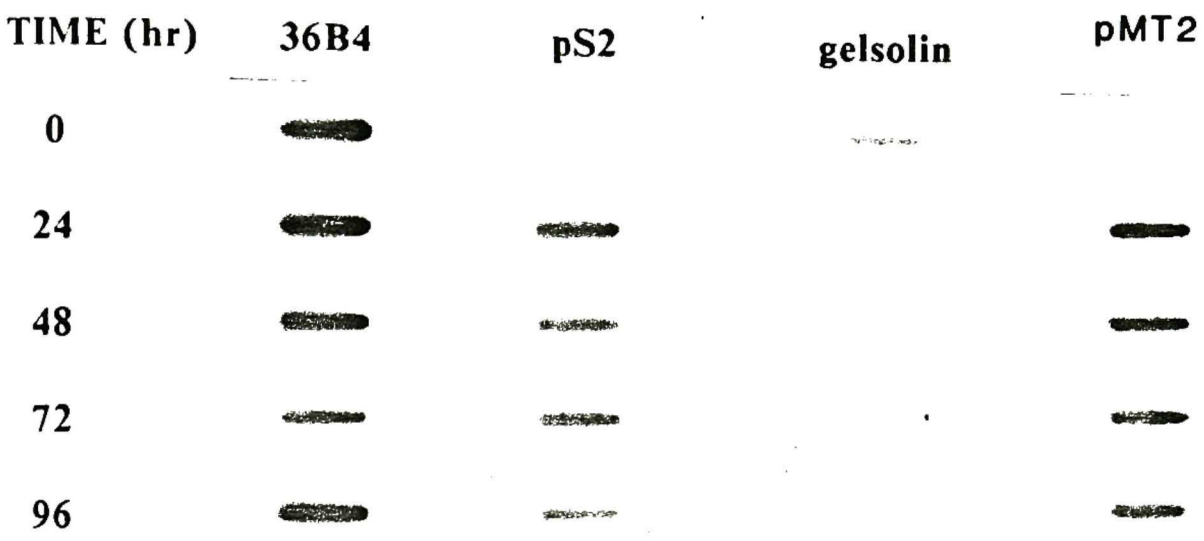


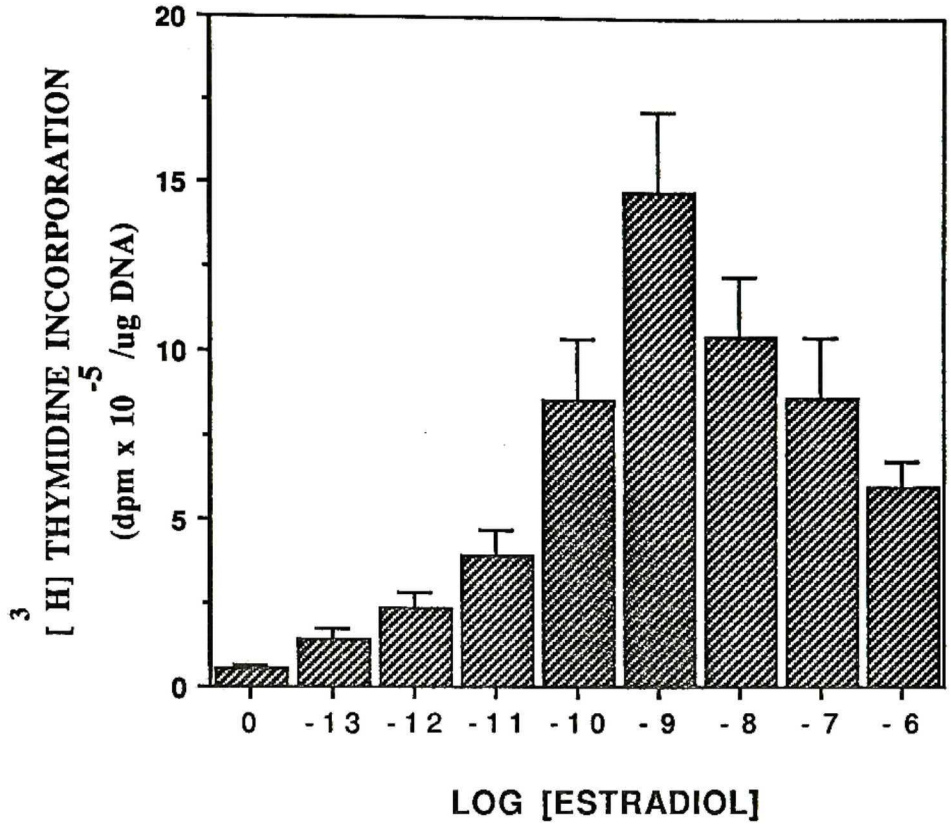
Figure 17. Effect of Estradiol on mRNA expression in MCF-7 Cells.

Radiolabeled DNA was prepared by the procedure of Feinberg and Vogelstein (1984). pS2, 36B4, and gelsolin were obtained as described in figure 8. pMT2 = pMT2A (4.4 kb fragment cloned into the EcoRI site of pGEM3Z).

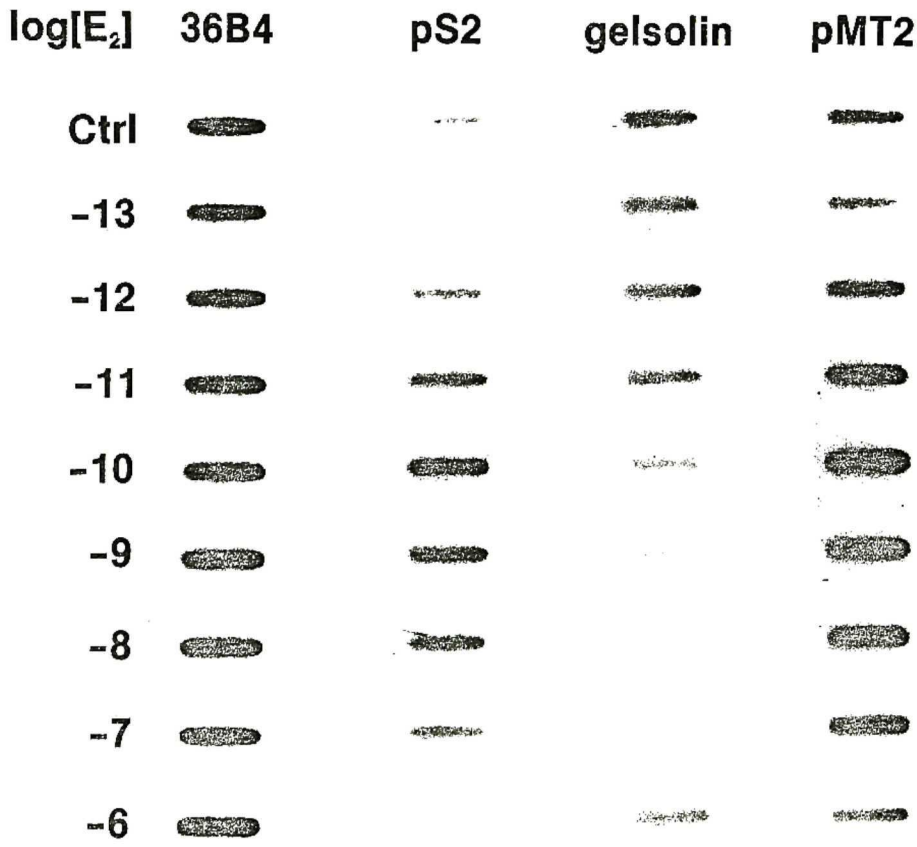
Figure 18. Effect of Varying Concentrations of Estradiol on MCF-7 Cells.

A. [^{3}H]Thymidine incorporation B. mRNA expression

A


[3H] THYMIDINE INCORPORATION
(dpm x 10-5 /ug DNA)
20
15
10
5
0
0
-13
-12
-11
-10
-9
-8
-7
-6
LOG [ESTRADIOL]

B


log[E2]
36B4
pS2
gelsolin
pMT2
Ctrl
-13
-12
-11
-10
-9
-8
-7
-6

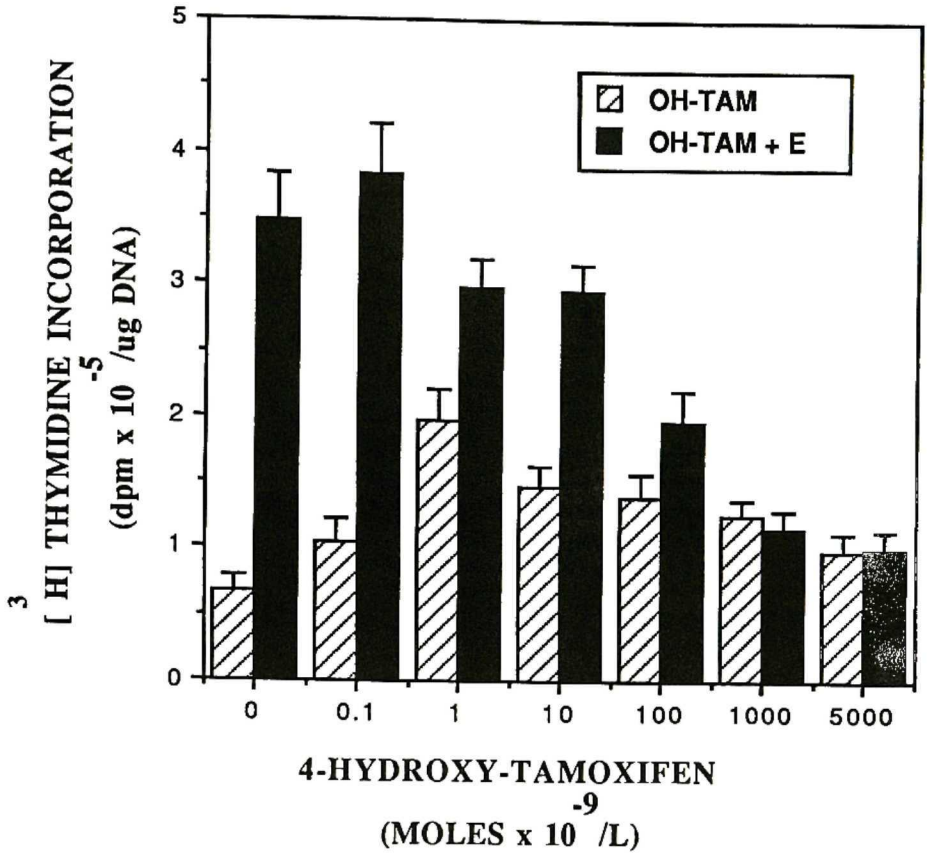
contrast, gelsolin mRNA levels declined progressively on treatment with 10^{-12}M and 10^{-11}M E_2, were not detectable at concentrations below 10^{-11}M, and reappeared at 10^{-6}M. pMT2 RNA could be detected in controls, but induction was maximal with 10^{-10}M E_2; 10^{-6}M E_2 produced a decrease in mRNA levels. Therefore concentrations of estradiol which are considered physiologically relevant, and which are known to act via the estrogen receptor, affected cellular growth and pS2 mRNA levels as anticipated and also altered the expression of pMT2 and gelsolin mRNAs.

The anti-estrogen 4-hydroxy-tamoxifen has often been employed to further demonstrate the specificity of phenotypic changes with respect to estrogen. OH-TAM and estradiol bind to estrogen receptor with approximately the same affinity. The results of twenty-four hours of treatment with varying doses of OH-TAM in the absence or presence of $2x10^{-10}$M estradiol are illustrated in figure 19. OH-TAM (10^{-9}M) alone stimulated [^{3}H]thymidine incorporation about 3-fold; other concentrations of OH-TAM were less effective. However concentrations of OH-TAM greater than 10^{-9}M also inhibited the stimulatory effect of estradiol. Indeed, 10^{-6} and $5x10^{-6}$M OH-TAM completely prevented the effect of E_2. OH-TAM similarly appeared to act as a partial agonist with respect to the regulation of pMT2 and pS2 expression since mRNA levels rose slightly and then declined at concentrations in excess of 10^{-7}M. Estradiol increased both pS2 and pMT2 mRNA levels. However the stimulation by estradiol was prevented by co-administration of 10^{-7} and 10^{-6}M OH-TAM. In contrast, OH-TAM had little effect on gelsolin mRNA levels although 10^{-10}M OH-TAM administration did increase expression slightly. Further, OH-TAM did not prevent the decline in gelsolin mRNA produced by estradiol. An increase in gelsolin mRNA was actually seen on co-treatment with 10^{-7}M OH-TAM and E_2. These results again suggest that the effects of estrogen on pMT2 gene expression are mediated via a classic estrogen receptor mechanism.

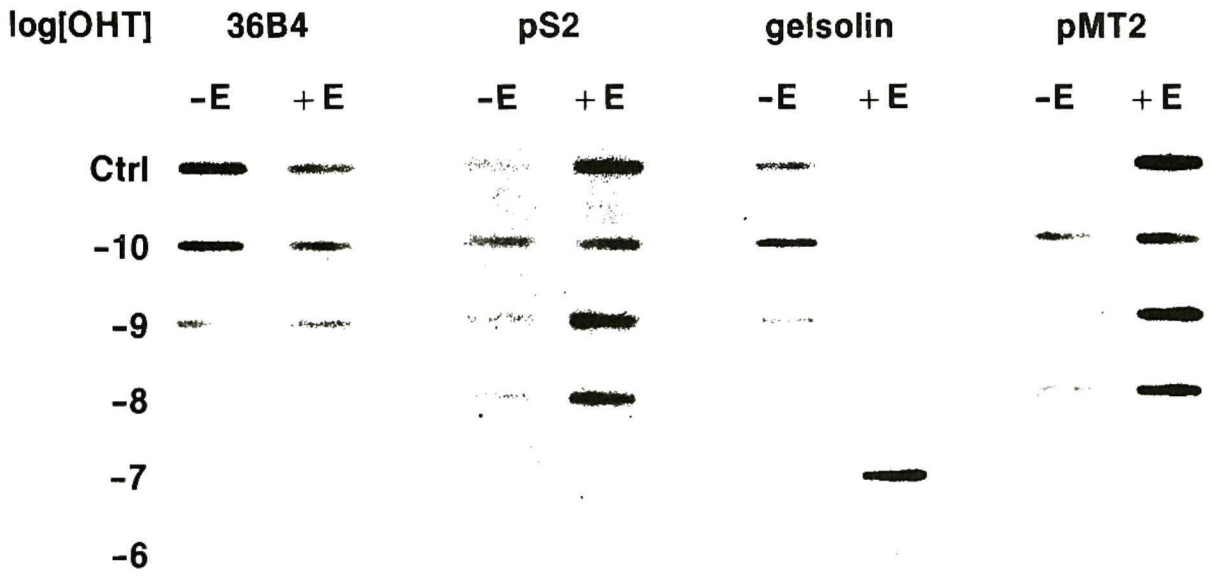
Figure 19. Effect of 4-Hydroxy-Tamoxifen and Estradiol on MCF-7 Cells.

A. [^{3}H]Thymidine Incorporation B. mRNA Expression

A


OH-TAM
OH-TAM + E
[3H] THYMIDINE INCORPORATION
(dpm x 10-5 /ug DNA)
0
1
2
3
4
5
0
0.1
1
10
100
1000
5000
4-HYDROXY-TAMOXIFEN
(MOLES x 10-9 /L)

B

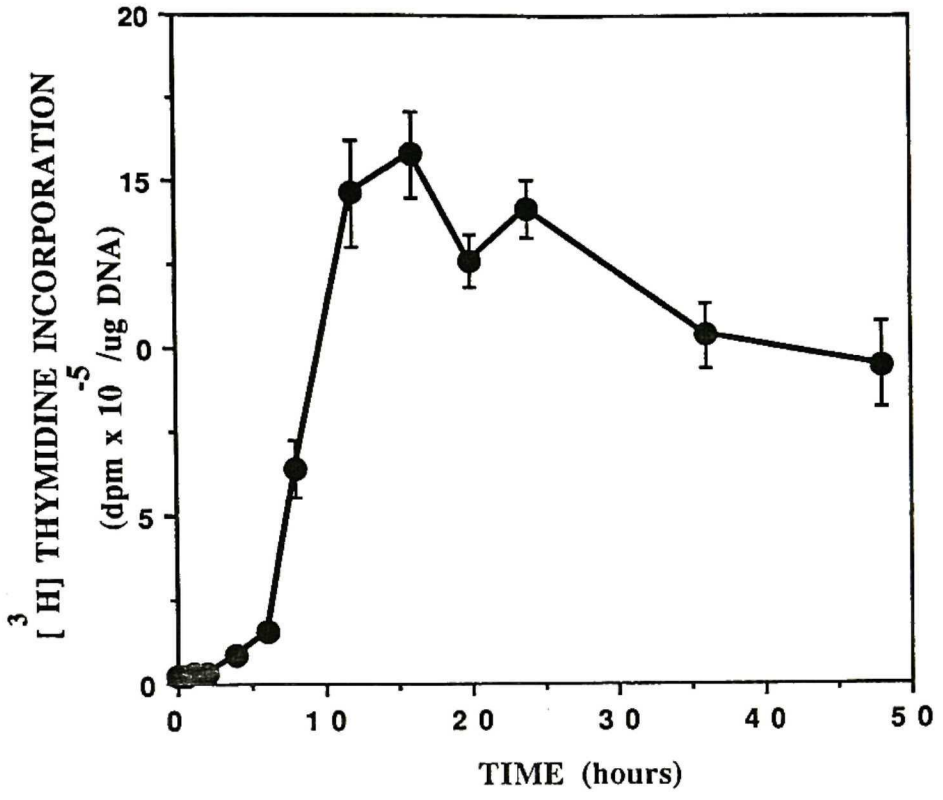

log[OHT]
36B4
pS2
gelsolin
pMT2
-E
+E
-E
+E
-E
+E
-E
+E
Ctrl
-10
-9
-8
-7
-6

In order to examine the relationship between pMT2 gene expression and the proliferation of MCF-7 cells, we then studied the effect of 10^{-9}M E_2 on [^{3}H]thymidine incorporation and mRNA expression over a 48 hour time period (figure 20A, 20B). [^{3}H]Thymidine incorporation began to rise after five hours, was maximal at twelve to twenty-four hours, and declined slightly thereafter. pS2 mRNA levels began to rise at one hour, were maximal at twelve hours, and remained elevated thereafter. Gelsolin levels decreased after one hour and were not detectable after eight hours. pMT2 mRNA expression increased within thirty minutes, continued to rise, and was maximal at eight hours. Levels then decreased to approximately one-third of the maximum value for the next thirteen hours, but appeared to increase again at the twenty-four hour time point. These data are graphically represented in figure 21. It is clear that the induction of pMT2 mRNA levels correlated closely with the stimulation of DNA synthesis. Further the temporal pattern of pMT2 gene expression, although similar, is not identical with that of pS2.

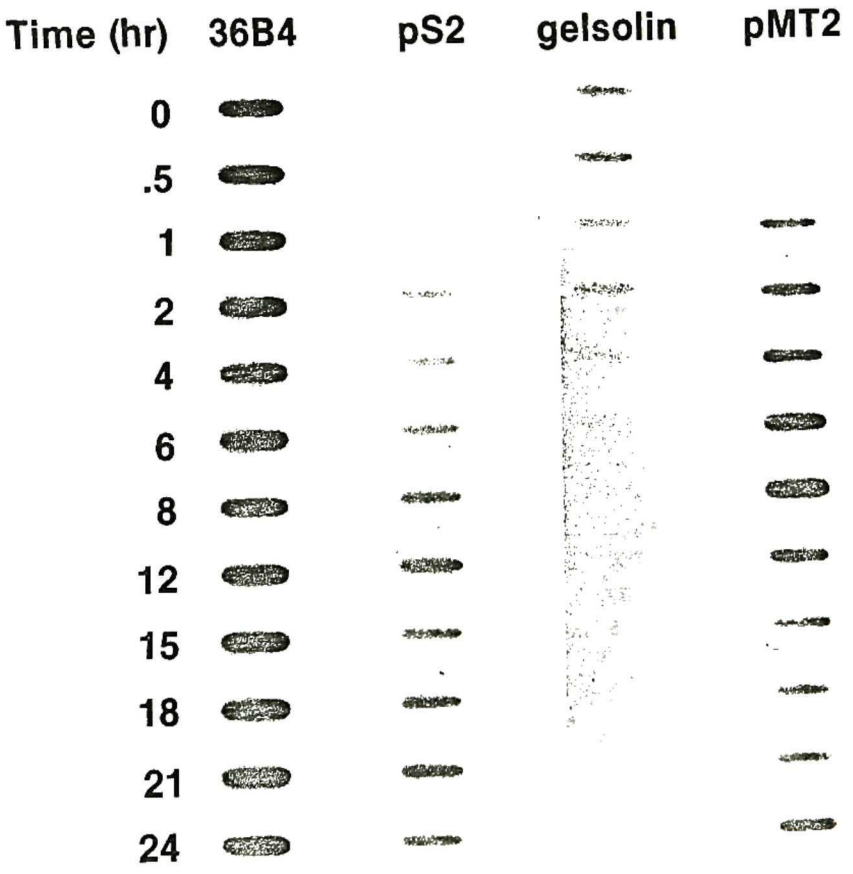
Clones pMT2A and pMT2B were then subjected to partial sequence analysis as described in Materials and Methods. Two long open reading frames (fifty-six amino acids for clone pMT2B sequenced with the SP6 primer; seventy-six amino acids in clone pMT2A sequenced with the T7 primer) were identified. No unequivocal translation initiation site was identified in any of the open reading frames. Further a search for functionally interesting sequences (leucine zipper, zinc finger, phosphorylation sites, ATP binding sites) using the IBI Pustell Sequence Analysis Program revealed only a number of possible N-glycosylation sites. However, pMT2 did not demonstrate significant homology with any gene registered in GENBANK. pMT2 therefore appeared to represent a previously unidentified gene. Also the pattern of pMT2 gene expression was characteristic for genes thought to be regulated by estrogen and the estrogen receptor.

Figure 20. Effect of Estradiol on [^{3}H]Thymidine Incorporation and mRNA Expression in MCF-7 Cells.

A.


20
15
0
5
0
0
10
20
30
40
50
TIME (hours)
^{3}[H] THYMIDINE INCORPORATION
(dpm x 10^{-5} /ug DNA)

B.


Time (hr)
36B4
pS2
gelsolin
pMT2
0
.5
1
2
4
6
8
12
15
18
21
24

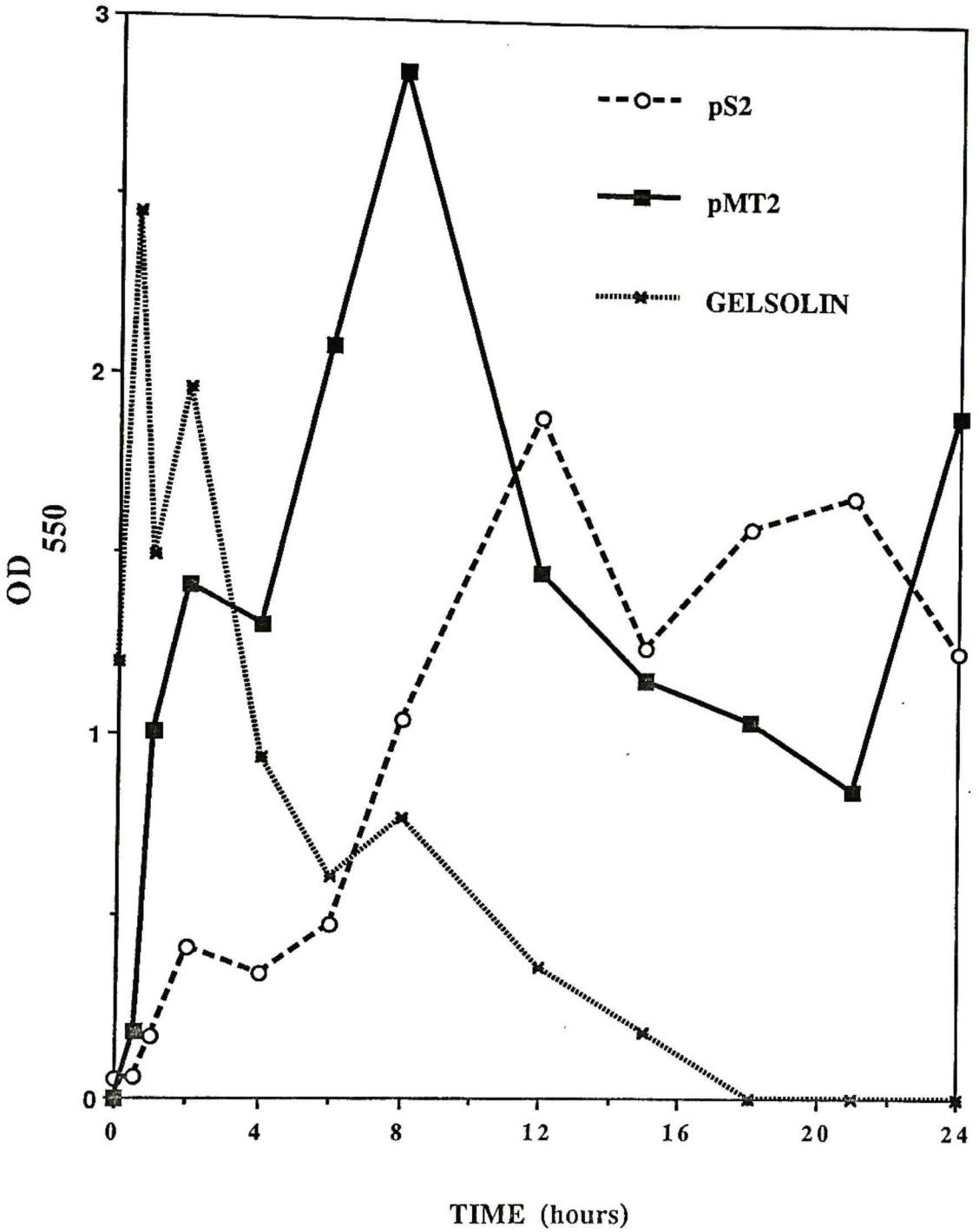


Figure 21. Effect of Estradiol on pMT2 Gene Expression.

The data in figure 20B were analyzed by scanning laser densitometry, and corrected for 36B4 expression (previously normalized against the value at time 0). For representational purposes corrected gelsolin values were multiplied by a factor of two.

DISCUSSION

Although estradiol appears to regulate the mRNA levels of several different genes, the role of any such gene products in the trophic response of tissues to hormone treatment remains unclear. The primary goal of this project was to generate a set of research tools which would permit subsequent studies on the mechanism of estrogen action. The molecular means by which estrogen alters gene activity, the molecular basis for the trophic response of various cell types to estradiol, and the development of potential markers or screening techniques for the appearance or progression of mammary tumors all represented potential targets for investigation.

This investigation required an experimental system in which estradiol comprised the sole variable regulating cell growth. The MCF-7 cell line, maintained under stringently controlled *in vitro* conditions, has proven to be of great utility in previous studies on the regulation of growth of mammary cancer, and also represents a highly characterized system both *in vitro* and *in vivo* (Horwitz *et al.*, 1975; Siebert and Lippman, 1982; Lippman *et al.*, 1986). Accordingly, rigorous experimental conditions eliminating all extraneous sources of estrogenic hormones were employed to examine the effect of estradiol on the growth of these cells. Figure 6 demonstrates the absolute requirement of MCF-7 cells for estradiol if any increase in cell number is to be sustained. When cells were maintained continuously in the presence of hormone, and were subsequently deprived of estradiol by sequential medium changes, the cell population stabilized in number and only resumed growth on readdition of hormone. The increase in cell number on readdition of estradiol was kinetically similar to that observed in cells continuously exposed to hormone. In addition, dramatic increases in [^{3}H]thymidine incorporation occurred within twelve hours of readministration of E_2 (figure 20A).

Elevations in total poly(A) RNA content observed in estrogen-treated MCF-7 cells are frequently associated with changes in the mRNA levels of specific gene products (Kasid *et al.*, 1986; Bronzert *et al.*, 1987; Brown *et al.*, 1984). Therefore, a series of previously cloned cDNAs were examined in the hope of developing a set of possible

controls for future experiments. The first of these, a cDNA known as 36B4 (courteously provided by Dr. P. Chambon, Instit. de Chimie Biologique Strasbourg, Strasbourg, France) was selected to internally standardize data. The nature of this gene and its cellular role are unknown; nonetheless, levels of 36B4 mRNA are reported to be essentially unaltered by estrogen treatment (Saceda *et al.*, 1989). Northern blots of mRNA preparations from estrogen-treated and estrogen-deprived MCF-7 cells confirmed this finding (figure 8).

The second of these cDNAs, pS2 (also provided by Dr. P. Chambon), has been an exceedingly useful positive control in a number of past studies. Rapid and dramatic increases in mRNA levels represent a primary hormonal response known to be directly mediated by the estrogen receptor (Roberts *et al.*, 1988). However the cellular role of the pS2 polypeptide is again unknown. Our data indicated consistent 20-100-fold inductions in pS2 mRNA expression in our experimental system.

The third gene represented a potential negative control (a gene product which is down-regulated by estrogen treatment), and was kindly made available by Dr. Carl Dieffenbach and Dr. Robert Silverman (Department of Pathology, USUHS). Gelsolin (MU-319, A319) is a cDNA clone encoding the mRNA for a member of a ubiquitous class of cellular proteins which regulate actin assembly and disassembly. The activity of gelsolin in non-proteolytically severing actin-actin contacts, promoting nucleation of new actin filaments, and blocking the positive ends of actin filaments, is known to be Ca^{++} dependent. Further, polyphosphoinositides differentially inhibit the severing activity of gelsolin. It had previously been observed that levels of gelsolin protein and mRNA increase upon differentiation of a variety of cell types (notably the conversion of embryonal carcinoma cell lines to visceral endoderm). On differentiation of embryonal carcinoma cells, previously disorganized actin (80% non-polymerized) is converted to well organized actin cables without measurable change in actual levels of actin proteins. The terminal differentiation of a mouse embryonal carcinoma line (PC-13) is associated with such phenotypic changes as loss of metastatic potential, increases in c-Fos protein, increased numbers of EGF receptors, and decreased levels of type IV collagen (Dieffenbach *et al.*, 1989). Gelsolin mRNA and protein levels also increase during differentiation of PC-13

cells following treatment with retinoic acid, dibutyryl cAMP, and 3-isobutyl-1-methylxanthene (Dieffenbach *et al.*, 1989). Gelsolin may thus function in mediating the dynamic equilibrium of the actin cytoskeleton, influencing cell shape, movement, secretory activity, etc. (Matsudaira and Janmey, 1988).

Several observations suggested that gelsolin might play a functional role in MCF-7 cells as well. The morphology of these cells is profoundly affected by a variety of culture conditions, including the presence or absence of estrogenic hormones (Pourreau-Schneider *et al.*, 1984; Pourreau-Schneider *et al.*, 1986). Most established mammary cancer cell lines, including MCF-7, stain diffusely for actin filaments (Corschellas *et al.*, 1987). Estrogen-responsive cells stimulated with estradiol display a prominent membrane 'ruffling', associated with the appearance of abundant microvilli, pseudopodial protrusions, actin-rich microfilamentous structures, and increased pinocytotic activity (Sapino *et al.*, 1986; Sica *et al.*, 1984). Tamoxifen (TAM, 10^{-6}M) produces similar morphological changes despite decreasing cellular proliferation . Unfortunately these previous experiments were conducted in the presence of phenol red.

In fibroblasts microinjection of the human H-*ras* protein (Bar-Sagi and Feramisco, 1986) or of $P34^{cdc2}$ kinase (Lamb *et al.*, 1990) results in actin filament redistribution and membrane ruffling. Treatment with EGF and PDGF also produces actin reorganization, coincident with marked increases in surface membrane ruffles and fluid phase pinocytosis (Paulsson *et al.*, 1986). E_2 increases MCF-7 cell adhesion, chemoinvasion, and migration in the presence of laminin, whereas v-Ha-*ras*-transformed MCF-7 cells constitutively express these same activities and are not affected by E_2. Such alterations in cellular motility, interactions with basement membrane, and secretion of proteases affecting extracellular matrix proteins could certainly be related to cytoskeletal changes. In view of the responses of MCF-7 cells to mitogenic stimuli (i.e. EGF, E_2), and the changes in *ras*-transformed MCF-7 cells (Kasid *et al.*, 1985), it seemed possible that estradiol might affect the activity and/or levels of gelsolin mRNA or protein. Treatment of MCF-7 cells with estrogen clearly decreased gelsolin steady-state mRNA levels (figure 7). This gene product thus appearred to constitute a suitable negative control in our experimental system.

The application of the following criteria permitted a stringent evaluation of the mRNA preparations to be employed in the construction of a cDNA library: 1. increased [^{3}H]thymidine incorporation; 2. increased levels of pS2 mRNA; 3. unchanged levels of 36B4 mRNA; 4. decreased levels of gelsolin mRNA. The conditions under which a given mRNA preparation was considered to derive from an estrogen-treated or estrogen-deprived population of cells are shown in table 6.

The next phase of this study required construction of a cDNA library whose population of recombinants mimicked the population of template mRNAs. Hybridization kinetics confirmed that the composition of the library (number of different species, molecular size, and frequency of individual species) indeed reflected that of the parent E+ poly(A) RNA template. A wide variety of cells and tissues have been found to contain betwen 10,000 and 30,000 different mRNA sequences (Woods *et al.*, 1980; Sargent and Dawid, 1984). Total poly(A) RNA sequence complexity in MCF-7 cells treated with E_2 was calculated as approximately 10,000 to 13,000, in good agreement with other reports. On the basis of the hybridization analyses, screening of 150,000 recombinants would permit a 95% chance of isolating a specific clone for an estrogen-regulated low abundance mRNA (table 9). The E+ cDNA library constructed in λgt10 comprised approximately 10^6 recombinants (table 7) and was therefore adequate for this purpose.

Detection and isolation of clones entailed three successive rounds of differential screening with radiolabeled cDNA synthesized from poly(A) RNA templates (prepared from either estrogen-treated or estrogen-deprived cells). Each probe was hybridized against excess E- poly(A) RNA to a R_0t value of 42. This procedure ensured that approximately 90% of low abundance cDNA species, as well as virtually all moderate and high abundance cDNAs common to both E+ and E- cDNAs, would be eliminated from the radiolabeled cDNA population. Thus filters screened with an E- cDNA should primarily reflect a non-specific background, whereas duplicate filters screened with E+ radiolabeled cDNA should display a higher number of positive signals (figure 13).

Clones appearing differentially on the E+ filter were then selected from the original plate and rescreened using a second set of selected cDNAs. Nineteen clones were isolated on the third and final round of screening (data summarized in table 12). Dot blots of

denatured λ cloned DNA hybridized with either an unselected E+ or E- cDNA confirmed that nine of the nineteen were indeed differentially recognized by the E+ cDNA. DNA mini-preparations of the nine clones were subsequently treated with EcoRI and inserts were isolated by electrophoresis on low-melting agarose. A matrix dot hybridization of all nine cloned λ DNAs versus all corresponding radiolabeled inserts indicated the existence of four distinct molecular species, one of which (pMT2) was represented six times (figure 15).

Although pS2 was not identified in the final nineteen clones, pS2 cDNA had clearly not been excluded from the E+ selected radiolabeled cDNA since both selected and unselected E+ radiolabeled cDNA hybridized efficiently to denatured pS2 DNA. As anticipated, gelsolin, a relatively non-abundant mRNA in MCF-7 cells which is down-regulated by estrogen, was not detected in either the selected or unselected E+ radiolabeled cDNA. In contrast, 36B4, which represents a moderately abundant species common to both E+ and E- mRNA populations, was present in the unselected but not the final selected E+ radiolabeled cDNA. These results suggested that elimination of common species by hybridization was both efficient and selective.

It appeared possible that any one of the clones could represent either pS2 or progesterone receptor since both of these genes are highly responsive to estrogen treatment. Although none of the nineteen clones obtained in the final round of screening proved to be pS2, one clone (#42) selected during the first round did in fact hybridize to radiolabeled pS2 DNA. This clone had not been chosen for further analysis since the strength of signal was judged to be relatively weak and indistinct. In addition, subsequent hybridization of the cDNA matix dot blot with radiolabeled progesterone receptor DNA (kindly provided by Dr. Bert O'Malley), confirmed that one of the final four cDNA clones (pMT1) encoded progesterone receptor. This result was considered reassuring since a known positive control had appeared in the final selection, and this mRNA represented a non-abundant mRNA species.

The data on these clones are summarized in table 10 and the Northern blots are seen in figure 15. Progesterone receptor (pMT1) was encoded by multiple species, notably 4.5 and 5.2 kb. The pMT1 cDNA was considerably smaller than the mRNA for PR. However several laboratories (Jeltsch *et al.*, 1990; Wei *et al.*, 1988; Read *et al.*, 1988) have reported

the existence of multiple PR mRNA species ranging in size from 1.8 to 11 kb depending on species and hormonal treatment. Variation has been attributed to post-transcriptional processing (alternative splicing, different polyadenylation sites, and 5' truncation). The other clones encoded single mRNA species of varying abundance. pMT3 encompassed 1.2 of the 1.7 kb parent mRNA species while pMT4 appeared to be a full length cDNA. The mRNA for pMT2 appears quite rare, and the cDNA for pMT2 was considerably smaller than its message (6.7 vs 11 kb). The observation that six of the final nine clones were identified as pMT2 despite relatively low levels of mRNA expression is especially intriguing.

pMT2 was subsequently subcloned into pGEM3Z as two separate EcoRI inserts of approximatedly 4.5 and 2.2 kb and subjected to partial sequence analysis. A search of Genbank indicated that no known cloned gene corresponded significantly with sequences represented in either insert. Various β-globin genes and pseudogenes presented the closest relationships (<42 percent homology). Analysis of the sequenced regions for a possible translation product did not reveal an unequivocal translation initiation site. Since the total insert size was approximately 6.7 kb and the mRNA species was 11 kb, it is possible that only the 3' and/or 5' untranslated regions were represented. Another possibility is that the 11 kb message was an unspliced precursor and the sequenced regions corresponded to introns. However, since the same cDNA appeared six times in the final nine clones, and since the digestion with EcoRI endonuclease produced virtually identical fragments of approximately 2.2 and 4.5 kb in all six, this possibilty is remote. Nonetheless, it is clearly an imperative to rescreen the original library or another library for the full length cDNA clone.

The fourth phase of this investigation involved verification that these clones truly represented estrogen-induced mRNA species. Increases in mRNA levels were seen upon long term estrogen treatment (figure 15). A series of additional experiments illustrating the response of pMT2 to estradiol and to the anti-estrogen 4-hydroxy-tamoxifen are shown in figures 17-21. Twenty-four hours of treatment with estradiol increased the steady-state levels of pMT2 at physiological concentrations of hormone (figure 18). 10^{-10}M E_2 maximally induced pMT2 and pS2 gene expression but repressed the expression of gelsolin

mRNA . pNR-2 (pS2) and pNR-1 have been reported to respond to estradiol with half-maximal inductions at approximately $5x10^{-10}$M and $2x10^{-11}$M respectively (May and Westley, 1987). Conversely, higher levels of estradiol decreased pMT2 (10^{-7}M and 10^{-6}M E_2) and pS2 gene expression (10^{-6}M E_2), and gelsolin mRNA again became detectable (10^{-6}M E_2). The effects of high levels of estradiol have been difficult to predict in various test systems. For example, 10^{-6} to 10^{-7}M estradiol can either stimulate or inhibit MCF-7 cell growth depending on the precise experimental conditions (Lippman *et al.*, 1976; Kasid *et al.*, 1985). Responses to pharmacological concentrations of estradiol (>10^{-6}M) have frequently been dismissed as reflecting non-specific, and possibly toxic, effects of a lipophilic molecule on cellular membranes and the mitotic spindle; however, some effects of high concentrations of steroids are restricted to estrogens, are stereospecific and are dependent on the presence of estrogen receptor (Reddell and Sutherland, 1987). High dose estrogen treatment has been successfully employed in the endocrine therapy of ER positive breast cancer patients who have become resistant to anti-estrogen (tamoxifen) treatment (Seibert and Lippman, 1982). The effect of high concentrations of estrogen may indeed reflect an unknown, yet highly specific, mechanism of action.

OH-TAM acted as a partial agonist with respect to both pS2 and pMT2 gene expression and incorporation of [^{3}H]thymidine into DNA (figure 19). 10^{-10}M OH-TAM alone slightly increased mRNA levels and [^{3}H]thymidine incorporation, and concentrations of OH-TAM in excess of 10^{-8}M anatagonized the action of estradiol. However, the effect of anti-estrogens has been reported to vary greatly depending on the experimental system. For example, OH-TAM may be a partial agonist with respect to pNR-1 since concentrations of 10^{-10} to 10^{-9}M increase mRNA levels to 80% of those observed on treatment with estradiol. In this case, neither OH-TAM nor TAM appeared to antagonize the effect of estradiol. In contrast, OH-TAM has been reported to increase pS2 gene expression to only 10% of the level seen with estradiol, and a 10-fold molar excess of OH-TAM does antagonize the effect of estradiol (May and Westley, 1987). It is interesting that 10^{-10} M

OH-TAM alone increased the expression of gelsolin. Moreover the combination of 10^{-7}M OH-TAM and $2x10^{-10}$M estradiol also increased gelsolin mRNA levels. This response is difficult to explain solely on the basis of competition for ligand binding sites on the estrogen receptor. The differential response to estrogens and anti-estrogens may reflect variation in the promoter environments of different genes; in addition, OH-TAM-receptor complex may not be structurally or functionally identical with E_2-receptor complex. Since ER binds to EREs as a dimer, and since the possibility of heterodimer formation exists, the regulation of gene expression by estrogen receptor may be much more complex than originally envisioned. Also certain responses to hormones may be secondary to other cellular regulatory events rather than being mediated directly by ER.

The effect of estradiol on pMT2 gene expression was evident within one hour of hormone treatment; maximum induction occurred at eight hours (approximately at the same time [^{3}H]thymidine incorporation was maximal), and levels of mRNA subsequently declined (figure 21). However mRNA levels consistently remained above controls at all later time points between twelve and twenty-four hours. In agreement with other reports, pS2 mRNA was detectable at one hour and levels increased steadily thereafter. Gelsolin mRNA gradually decreased and could not be detected after eight hours. The normalized data indicated that pS2 and pMT2 differ most obviously in the relative decline in pMT2 after twelve hours of exposure to E_2.

Cumulatively these data suggest that the effect of estradiol on pMT2 is a receptor-mediated event. At this time we have no evidence indicating that stimulation of pMT2 gene expression represented a primary response to hormone, nor is the molecular mechanism of this response (transcriptional, post-transcriptional) known. Further, despite the parallel in [^{3}H]thymidine incorporation and pMT2 gene expression, it is impossible to state that this particular clone is involved at any level in mediating the trophic response of these cells.

In theory, any of the three clones we have identified could regulate cell growth by a variety of mechanisms. Oncogenes, for example, appear to function at several levels of cellular control and can be cited as examples of potential regulatory molecules at each of the steps detailed below.

1. Most immediately, the protein product of one of our clones could be a growth factor or a growth factor receptor (reviewed by Ullrich and Schlessinger, 1990). TGF-α is secreted by human mammary cancer cells and is itself an agonist for the EGF receptor (c-*erb*B-1), thus establishing a stimulatory autocrine loop. The natural ligands for two putative oncogene products closely related to the EGF receptor (c-*erb*B-2 and c-*erb*B-3) have yet to be identified. Likewise, the proto-oncogene c-*met* probably encodes a tyrosine kinase receptor for an unknown ligand.

2. On a second level, our protein could provide a link between a specific membrane receptor and the subsequent intracellular events leading to cell division. Most hormones and other extracellular stimuli transmit their signals by one of three mechanisms: the cyclic AMP pathway; the phosphatidyl-inositol/Ca^{++} pathway; or by ion channels. One such group of mediators are exemplified by the G proteins which function in a variety of systems as signal transducers (Stryer and Bourne, 1986). The oncogene prototypes for this group are the various proteins comprising the *ras* family. Ras is essential in mediating the cellular response to various mitogenic stimuli and is required for tranformation by oncogenes with tyrosine kinase activity. McCormick and colleagues (McCormick, 1989) have identified a protein termed GAP (GTPase activating protein) which interacts directly with Ras. GAP may provide a mechanism coupling Ras to other membrane proteins. For example, Ras·GAP complexes block K+[Ach] channels in atrial cells. It appears that the complex acts upstream of the channels either to prevent coupling of muscarinic receptors to endogenous G_k proteins or to inhibit the association of $G_{k\alpha}$ with its $\beta\gamma$ units (Yatani *et al.*, 1990).

Regions of Ras dispensable for transformation are also dispensable for GAP interaction, but whether GAP regulates Ras or vice versa is not entirely clear (Vogel *et al.*, 1988). Several recent lines of evidence suggest that GAP catalyzes the conversion of active GTP ·Ras to inactive GDP ·Ras, possibly by increasing the intrinsic GTPase activity of the Ras protein itself. It has been postulated that growth factor-occupied receptors (many of which manifest kinase activity) phosphorylate GAP, preventing the Ras·GAP interaction and leaving Ras in an activated state. PDGF and EGF (but not FGF) increase the tyrosine

phorphorylation of GAP (Kaplan *et al.*, 1990; Hall , 1990). Mutations in GAP, Ras, or receptors could thus result in a permanently activated Ras protein and uncontrolled cellular proliferation.

However, the effect of mutations in various domains of Ras are also consistent with GAP acting as a Ras effector protein. In general, cells transformed with *ras* oncogenes display a lowered requirement for growth factors and antibodies to Ras block the action of several growth factors. After PDGF treatment, GAP physically associates with PDGF receptor forming a complex with the oncogene product Raf-1, phospholipase C-γ (PLC-γ), and phosphatidyl-inositol-3-kinase (PI-3-kinase). Cells transformed with activatated c-Ha-*ras* are defective in response to PDGF; i.e. PDGF receptor no longer increases phospholipase C (PLC) and phospholipase A_2 (PLA_2) activities. In such cases, GAP does not associate with the PDGF receptor and is not phosphorylated. However, the PDGF receptor retains tyrosine kinase activity and still complexes with PLC-γ and PI-3-kinase. It is possible that GAP provides a bidirectional link between growth factor receptors and the p21-*ras* family of proteins.

The downstream effectors of Ras are not identified. Ras appears to function in several capacities, and the sequence variability associated with specific regions in different *ras* proteins may reflect interactions with several different downstream effectors. It is very clear that *ras* proteins control adenylate cyclase activity in yeast and therefore may indirectly regulate the kinase activity of protein kinase A. Mammalian and yeast *ras* genes are functionally interchangeable *in vivo*, but Ras apparently does not act on adenyl cyclase in metazoan systems. Investigators have since identified a yeast gene termed CAP1 whose protein product is a 70 kd protein which complexes with adenyl cyclase and which appears to mediate the action of Ras on this enzyme (Fedor- Chaiken *et al.*, 1990; Field *et al.*, 1990). Investigations are now proceeding to determine whether a similar protein couples metazoan Ras to its effector systems.

It has been suggested that a second G protein might mediate the effects of several growth factors thought to interact with the phosphatidyl-inositol/Ca^{++} pathway. The inital consequences of many growth factor receptor-ligand interactions (i. e. PDGF, EGF) include tyrosine-specific protein phosphorylations, inositol lipid hydrolysis, activation of

Na^+/H^+ exchange, and rapid alterations in cellular Ca^{++} pools. The folowing sequence of events has been suggested. Initially a putative G protein activates PLC. PLC subsequently cleaves membrane phosphoinositols to yield inositol phosphates (notably IP-3) and 1, 2-diacylglycerol (DAG). The inositol phosphates trigger Ca^{++} mobilization. Ca^{++} and DAG then activate the Ca^{++} phospholipid-dependent protein kinase (protein kinase C; PKC). PKC acts as a serine/threonine kinase. A large body of evidence suggests that PKC provides negative feedback control on various cell signalling processes (Nishizuka, 1986). For example PKC decreases the IP-3-induced elevation of intracellular calcium and phosphorylates EGFR and c-*met* protein on serine, decreasing the high affinity binding and the autophosphorylation on tyrosine of these receptors (Gandino *et al.*, 1990). However protein kinase C is an inclusive term for at least seven subspecies which display distinct tissue distribution patterns and kinetic profiles (Nishizuka, 1988). As is the case for Ras and for protein kinase A (PKA), the downstream effectors in this pathway are not identified. Presumably, PKC phosphorylates and alters the function of other intracellular proteins, eventually affecting cellular proliferation.

It should be noted that a number of investigations indicate that growth factor receptors such as EGF and PDGF might not function solely via the phosphatidyl-inositol pathway and PKC. The initiation of DNA synthesis and EGFR down-regulation are not coupled to the activation of PKC in several cell systems. For example, the ability of phosphatidyl-choline-phospholipase C (PC-PLC) to increase DNA synthesis in Swiss 3T3 cells is independent of protein kinase C (Han *et al.*, 1990). Larradera *et al.* (1990) suggest that phosphatidyl-choline might be a critical alternative source of IP-3 and DAG. It is interesting that cells transformed with v-Ha-*ras* show a dramatic stimulation of this degradative pathway.

3. One of our clones could also encode a protein corresponding to a group of oncogenes which may function as mediators distal to the plasma membrane. These proteins are both substrates for phosphorylation and themselves manifest kinase activity. For example, the c-*raf* gene family encodes serine/threonine protein kinases which associate with a variety of receptors (i.e. PDGF receptor) and which, after auto-phosphorylation on tyrosine, translocate to the nucleus (Kolch *et al.*, 1990). Whether c-

raf proteins regulate transcriptional activity directly or via a series of downstream effectors is not known. c-Src, a tyrosine specific protein kinase which is associated with the cytoplasmic face of the plasma membrane as well as other cellular membranes, may also function at this level (Shenoy *et al.*, 1989). The protein is itself phosphorylated on threonine, serine, and tyrosine residues. Interestingly, v-Src phosphorylates GAP. The identification of the cellular factors which regulate the kinase activity of v-Src may thus clarify a functional link between these tyrosine kinase oncogenes and various G proteins (Hall, 1990). In addition, c-Src is an apparent candidate for phosphorylation by $p34^{cdc2}$, one of the cell cycle genes identified in yeast. This subject will be discussed in more detail below.

4. Our unknown protein molecule could also interact with DNA and alter the transcriptional activity of specific genes. For example, GCN4, TFIIIA, and the steroid hormone receptor superfamily represent ' zinc finger' proteins; the prototype oncogene *fos* can be taken to represent the so-called 'leucine zipper' group (Introduction, pages 4 and 25). Fos is a transcriptional regulatory protein which may well govern a set of secondary changes in gene expression necessary for cell cycle progression (activation of such genes as thymidine kinase, histones, etc.). *Fos* protein and mRNA expression are themselves closely linked to cell cycle progression. Evidence suggests that c-Fos is induced by TPA (via PKC), by PDGF (possibly via another intracellular mediator) and by serum (Siegfried and Ziff, 1989). The promoter region of c-*fos* also includes sequences (serum response element, SRE; cAMP response element, CRE) linked to the phosphatidyl-inositol/Ca^{++} and cAMP pathways respectively (Gilman, 1988). The SRE is necessary and sufficient for response to both PKC-dependent and independent intracellular signalling pathways, but not for response to the cAMP pathway. The PKC-independent pathway is sensitive to both serum and PDGF. It has since been reported that the *jun* and *fos* proto-oncogene products efficiently activate transcription from both SRE and CRE promoter elements (Sassone-Corsi *et al.*, 1990). Curiously, the CRE cannot be activated by TPA. It is exceedingly interesting that three major intracellular transduction systems appear to converge in the regulation and action of a single critical gene.

5. One of the cDNA clones may define a gene which is directly required for cell cycle progression. The various cdc gene products, the components of MPF (Introduction, pages 27-28), DNA polymerases, the host of enzyme activities which vary with the cell cycle (dihydrofolate reductase, thymidylate synthetase, etc.), and the structural proteins which affect such processes as mitotic spindle disassembly are possible candidates in this capacity. Although a particular oncogene does not present a viable model at this level of growth regulation, some recent discoveries relating to MPF have yielded startling insight into cell cycle events (reviewed by Lewin, 1990). It is important to note that once MPF is activated all the subsequent structural changes associated with mitosis can occur via a phosphorylation cascade and without additional protein synthesis. MPF is comprised of a serine/threonine kinase ($p34^{cdc2}$) and two closely related cyclins (B1 and B2). Cyclin B2 is a substrate for phosphorylation by the proto-oncogene product c-Mos (*Roy et al.*, 1990). The possible substrates for $p34^{cdc2}$ include $pp60^{c\text{-}src}$, H-1 histone, cyclin B, nucleolin, and the lamins. The general function of all these substrates could include cytoskeletal rearrangements, nuclear envelope breakdown, and chromosome condensation (Moreno and Nurse, 1990). It is now established that the phosphorylation of lamin C and lamins B1 and B2 by $p34^{cdc2}$ results in nuclear envelope breakdown and chromosome condensation (Ward and Kirschner, 1990; Peter *et al.*, 1990). c-Src is also a substrate for $p34^{cdc2}$ during mitosis (Shenoy *et al.*, 1989; Morgan *et al.*, 1989). The close association of c-Src with various cellular membrane systems suggests a key role in mediating cytoskeletal and membrane structural changes associated with mitosis. $p34^{cdc2}$ thus seeems to function as a 'trigger' in regulating cell cycle progression. As noted in the introduction to this dissertation, similar 'triggers' are suspected to regulate the transition of cells through G1; as yet, however, the critical events in this phase of the cell cycle are not defined.

An important feature of these possible mechanisms of action is the degree of interaction of intracellular signalling systems. Insulin, for example, produces extremely complex phenotypic changes on target cells. The insulin receptor is thought to be coupled to both the cAMP and phosphatidyl-inositol/Ca^{++} pathways (Espinal, 1987). In addition,

positive and negative feedback occurs between the various levels of control as well as within a given level (i.e. down-regulation of growth factor receptors by other membrane receptors; complex gene regulation patterns exhibited by *fos*, etc.). It is also clear that specific post-translational protein phosphorylations are associated with the control of cellular proliferation. However, the fact that a particular protein is phosphorylated during a particular period of the cell cycle, or is a substrate for phosphorylation by another protein, can be overinterpreted. Many kinases have a generalized action and a broad range of substrates. It is critical to establish the specific sites of phosphorylation and define the functional and sequence specificity both *in vivo* and *in vitro*.

Unfortunately the limited sequence data available on pMT2 does not allow an accurate comparison with any of the above prototype regulatory molecules. Sequence analysis did not reveal homologies with the zinc finger or leucine zipper regions of transcriptional regulatory proteins or suggest GTP or ATP binding sites. One or two possible phosphorylation sites and several N-glycosylation sites were detected, but these observations cannot be considered significant at this time.

In conclusion, although these investigations are preliminary, it would appear that this project has been successful in that at least one cDNA which is estrogen-regulated has been cloned. It is now possible to investigate the regulation of this gene at several levels, and a number of distinct projects can be envisioned.

Mechanistic questions (primary versus indirect response to hormone; transcriptional vs. post-transcriptional regulation) can certainly be addressed. The effect of various estrogen agonists and antagonists on gene expression in anti-estrogen resistant MCF-7 mutants and in ER- vs. ER+ cell lines is readily determined. However, a definitive answer concerning the role of ER in mediating the stimulation of pMT2 gene expression will require cloning of the regulatory requences for pMT2 from a genomic library, subcloning into some plasmid construct, and co-transfection of ER- cells with an expression vector for ER. Nuclear run-on experiments and nuclease protection assays can differentiate between transcriptional and post-transcriptional effects on mRNA levels. Inhibitors of transcription such as actinomycin D and α-amanitin might also prove useful in this case.

Nonetheless, the identification of these clones and their functional role in regulating MCF-7 cell growth remains the primary question. It is tempting to speculate that any one of the clones could encode a growth factor or growth factor receptor such as c-ErbB (Downward *et al.*, 1984), a DNA-binding protein and transcriptional regulator such as c-Jun or c-Fos ((Ryder *et al.*, 1988), or some critical 'trigger' protein which regulates cell cycle transit as do the cdc genes in yeast (Nurse, 1985). Sequencing and comparison of the coding regions of our cDNAs with other proteins would give some indication of such an identity. Also the differential appearance of mRNA encoded by these clones in malignant tissues might be construed as evidence for a possible oncogene or anti-oncogene. Although a genetic component undoubtedly contributes to the genesis of mammary cancer, a causal link to known oncogenes is not yet established, and isolation of a putative 'transforming' gene functioning in human breast cancer would be extremely significant.

Determination of the functional significance of these new clones in the trophic response of mammary cancer to estradiol is a clear experimental priority. Additional research projects could include screening of malignant versus benign mammary tissues for differential gene expression; use of anti-sense RNAs or antibodies against the putative protein product to block or alter cell growth; and examining regulation of the cDNAs by other agents known to affect MCF-7 cell growth (EGF, TGF-α, TGF-β, IGF-1, IGF- 2, etc.). Unfortunately, most experiments which are biologically oriented will provide only correlative information further coupling gene expression and proliferative status. It will prove very difficult to definitively establish that any particular gene regulates cell growth using either biologically oriented experimental approaches or even such direct molecular biology techniques as transfection. For example, if it were found that that expression of a given clone is sufficient but not essential for cell proliferation, this particular gene may be functionally redundant, as has been suggested for the cyclins. Conversely, if it were observed that clone expression is essential but not sufficient for cellular proliferation, other genes may be necessary as well. Extending the possibilities even further, if estrogen treatment were either sufficient but not essential, or essential but not sufficient, for stimulation of either clone expression or cell proliferation, multiple regulatory elements may contibute to the control of this particular gene.

Decisions concerning the direction of future research programs will require careful thought. However, one mark of a successful research project is the genesis of either additional questions or the experimental means to address those questions. From that standpoint, the results of this study have been both intriguing and fruitful.

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